# Host-microbiota interactions in *C. elegans*: investigating the role of dietary glucose in the probiotic effects of *Bacillus subtilis* lacking ATP synthase

**by**

**Henry James Frédéric de Hauke Ancell**

A thesis submitted in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy

University of Warwick, School of Life Sciences

August 2018

**Table of Contents**

**List of Figures**

**List of Tables**

**Acknowledgements**

First and foremost, I would like to give special thanks to my supervisor, Dr Andre Pires da Silva. I really could not have asked for a better mentor. Thank you for your support and for giving me the freedom to explore my own ideas.

Thank you to the members of the Pires lab, both past and present, who I met along the way and who made time as a postgraduate so memorable. I am particularly indebted to Dr Sally Adams, who was a constant source of guidance and inspiration. I am also very grateful to Dr Anne-Claire Jacomin for all of her assistance in the lab.

I would like to thank my Ph.D advisory panel, Dr Ioannis P Nezis and Dr Emma Denham, for their scientific discussions and insights. I am thankful to Dr Denham in particular for her great experimental and technical help, and for her patient guidance throughout my Ph.D. Many thanks, too, to Dr Munehiro Asally and Dr Teja Sirec for their generosity during our brief collaboration.

Thank you to the team behind the Midlands Integrative Biosciences Training Partnership (MIBTP) for all of the skills they helped to foster, and to the MIBTP 2014 cohort with whom I shared the highs and the lows of Ph.D life. Thanks also to the BBSRC for their financial support.

Finally, I would like to thank my family for their unwavering encouragement and love.

**Declaration**

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

- The lifespan assays presented in Fig. 3. 3 and 3. 4 as well as Fig. 4. 7. The corresponding raw data are presented in Table A1, A2 and B8 and were collected by Alice Neilson during her Master's (MBio) project (Neilson, 2017). Graphs were generated by the author using this data.

- The experiments presented in Fig. 6. 7, 6. 8 and 6. 9. The data are given in Table D7, D8 and D9, and were collected by Said (2018) as part of her Master's (MBio) project. All graphical depictions and statistical analyses in this case were carried out by the author.

**Abstract**

The intestinal microbiota plays a critical role at the interface between nutrient uptake and host metabolism, and the bacterivorous nematode *Caenorhabditis elegans* presents a simple system in which to understand these interactions. The soil bacterium *Bacillus subtilis* enhances health and stress resistance in *C. elegans* compared to the standard diet of *E. coli*. Glucose is usually toxic to *C. elegans*, but a glycolysis-dependent *B. subtilis* 168 mutant lacking $F_1F_0$ ATP synthase (*B. s.* Δ*atp*) extends lifespan by up to 40% in the presence of 2% D-glucose, compared to animals fed on the master strain (*B. s.* MS). Glucose does not affect the lifespan of *C. elegans* fed on *B. s.* MS. This work presents two sets of novel findings; first, *C. elegans* fed on *B. s.* Δ*atp* in the absence (-Glu) and the presence (+Glu) of glucose displays characteristic signs of dietary restriction (DR), a highly conserved response to reduced nutrition without malnutrition. *B. s.* Δ*atp*(-Glu) delays development, improves health without longevity and regulates DR-related genes including PHA-4/FOXA. Extensive sporulation of *B. s.* Δ*atp*(-Glu) appears to contribute to these phenotypes. In response to glucose, this diet develops a rudimentary biofilm which is likely to obstruct feeding and underlie a coordinated DR response – including larval arrest, small body size, longevity and delayed egg laying. Second, when these issues are overcome by limiting biofilm development and preventing bacterial sporulation, the mutant diet improves lifespan and health in *C. elegans* probably through a separate mechanism that is not yet identified. These findings demonstrate that microbial metabolism can dramatically alter the effects of dietary glucose on host physiology and highlight important challenges associated with the provision of non-standard diets to *C. elegans*. Moreover, this diet-mediated uncoupling of lifespan from health and stress resistance offers an opportunity to uncover conserved molecular determinants of sustained health.

**Abbreviations**

| | |
|---|---|
| **2-DG** | 2-deoxy-D-glucose |
| **α-KG** | α-ketoglutarate |
| **Δ*atp*** | *B. subtilis* lacking the ATP synthase operon |
| **μl** | Microliter |
| **μm** | Micrometre |
| **μM** | Micromolar |
| **ACDH-1** | Acyl-CoA dehydrogenase-1 |
| **Acyl-CoA** | Acyl-coenzyme A |
| **ADR** | Axenic dietary restriction |
| **AGE** | Advanced glycation end product |
| **Ago** | Argonaut protein |
| **AMP** | Adenosine monophosphate |
| **Amp** | Ampicillin |
| **AMPK** | AMP-activated protein kinase |
| **ANOVA** | Analysis of variance |
| **ATP** | Adenosine triphosphate |
| **AU** | Arbitrary units |
| **Avid** | Age-associated vulval integrity |
| **BD** | Bacterial deprivation |
| **bDR** | Bacterial dietary restriction |
| **bp** | Base pair |
| ***B. s.*** | *B. subtilis* |
| **BSA** | Bovine serum albumin |
| ***B. subtilis*** | *Bacillus subtilis* |
| **CA** | Colanic acid |
| **cDNA** | Complementary DNA |
| ***C. elegans*** | *Caenorhabditis elegans* |
| **cfu** | Colony-forming units |
| **CGC** | *Caenorhabditis* Genetics Center |
| **Chlor** | Chloramphenicol |
| **conc** | Concentrated |
| **CoQ** | Coenzyme Q |
| **CSF** | Competence and sporulation stimulating factor |
| **DAF** | Abnormal dauer formation |

| | |
|---|---|
| **$_{dd}H_20$** | Double distilled $H_20$ |
| **DIC** | Differential interference contrast |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxyribonucleotide triphosphate |
| **DR** | Dietary restriction |
| **DSM** | Difco sporulation medium |
| **dsRNA** | Double-stranded RNA |
| ***E. coli*** | *Escherichia coli* |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EPS** | Exopolysaccharide |
| **Ery** | Erythromycin |
| **ETC** | Electron transport chain |
| **EtOH** | Ethanol |
| **FDR** | False discovery rate |
| **FOXO** | Forkhead box O |
| **FUdR** | 5-fluorodeoxyuridine |
| **GFP** | Green fluorescent protein |
| **-Glu** | Without 2% glucose |
| **+Glu** | With 2% glucose |
| ***gly-1*** | Glycogen synthase |
| **GMM** | Green master mix |
| **GST** | Glutathione S-transferase |
| **HIF-1** | Hypoxia-inducible factor 1 |
| **$H_2S$** | Hydrogen sulfide |
| **HSD** | Honest significant difference |
| **HSF-1** | Heat shock factor 1 |
| **HSP** | Heat shock protein |
| **HSR** | Heat shock response |
| **HYP** | Hypoxic conditions |
| **IDT** | Integrated DNA Technologies |
| **IF** | Intermittent fasting |
| **IGF-1** | Insulin-like growth factor-1 |
| **IIS** | Insulin/IGF-1-like signalling |
| **ILP** | Insulin-like peptide |
| **IPTG** | Isopropyl β-D-1-thiogalactopyranoside |
| **L** | Litre |

| | |
|---|---|
| **L1 – L4** | Larval stage 1 – larval stage 4 |
| **LAB** | Lactic acid bacteria |
| **lac** | Lactose |
| **LB** | Lysogeny broth |
| **LBA** | LB agar |
| **LDR** | Liquid dietary restriction |
| **M** | Molar |
| **MAPK** | Mitogen-activated protein kinase |
| **MDT-15** | Mediator-15 |
| **MG** | Methylglyoxal |
| **ml** | Millilitre |
| **MS** | Master strain |
| ***mtl-1*** | Metallothionein-1 |
| **NAC** | N-acetylcysteine |
| **NAD** | Nicotinamide adenine dinucleotide |
| **NADH** | Nicotinamide adenine dinucleotide-Hydrogen |
| **NEB** | New England Biolabs |
| **NGM** | Nematode Growth Medium |
| **NHR** | Nuclear hormone receptor |
| **NO** | Nitric oxide |
| **NOS** | Nitric oxide synthase |
| **Nrf** | NF-E2-related factor |
| **NRT** | No reverse transcriptase control |
| **NTC** | No template control |
| **OD** | Optical density |
| **PAGE** | Polyacrylamide Gel Electrophoresis |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PEG** | Polyethylene glycol |
| **Phleo** | Phleomycin |
| **$PIP_2$** | Phosphatidylinositol 4,5-bisphosphate |
| **$PIP_3$** | Phosphatidylinositol (3,4,5)-trisphosphate |
| **pL** | picoliter |
| **PM** | Paris medium |
| **PMK-1** | p38 MAPK |
| **PQ** | Paraquat |

| | |
|---|---|
| **qRT-PCR** | Quantitative reverse transcription PCR |
| **RISC** | RNA-induced silencing complex |
| **RdRP** | RNA-directed RNA polymerase |
| **RFP** | Red fluorescent protein |
| **RNA** | Ribonucleic acid |
| **RNAi** | RNA interference |
| **RNAseq** | RNA sequencing |
| **ROS** | Reactive oxygen species |
| **rpm** | Rotations per minute |
| **RT** | Room temperature |
| **sDR** | Solid dietary restriction |
| **SDS** | Sodium dodecyl sulfate |
| ***sigA*** | Sigma factor A |
| **siRNA** | Small interfering RNA |
| ***sod*** | Superoxide dismutase |
| **Spec** | Spectinomycin |
| **St. Dev.** | Standard deviation |
| **SREBP** | Sterol regulatory element-binding protein |
| **TAE** | Tris-acetate-EDTA |
| **TE** | Tris-EDTA buffer |
| **TGF-β** | Transforming growth factor-beta |
| **TOF-MS** | Time-Of-Flight Mass Spectrometry |
| **TOR** | Target of rapamycin |
| **trp** | Tryptophan |
| **TSAP** | Thermosensitive Alkaline Phosphatase |
| **unc** | Uncoordinated |
| **unconc** | Unconcentrated |
| **UPR$^{mt}$** | Mitochondrial unfolded protein response |
| **UV** | Ultraviolet |
| **VHL-1** | Von Hippel-Lindau tumor suppressor homolog-1 |
| **WT** | Wild type |

# 1 Introduction

## 1.1 What is ageing and why does it occur?

The phenomenon of ageing is characterized by loss of function, heightened vulnerability to disease and an increase in the risk of mortality over time (Fontana *et al.*, 2010). Evolutionary explanations for ageing are centred on the realization that the force of natural selection declines with age. Mutations with late-acting deleterious effects will accumulate over time, as natural selection is unable to purge them from the genome ('mutation accumulation', Medawar (1952)). Death as a result of extrinsic causes, including disease and predation, underlies the declining force of selection, as individuals are likely to succumb prior to the manifestation of the detrimental trait (Medawar, 1952). Similarly, certain late-acting detrimental alleles will be subject to positive selection if they enhance survival probability in early life ('antagonistic pleiotropy', Williams (1957)). Therefore, rather than being an evolved deterministic program, it is increasingly understood that non-adaptive destabilizing forces drive ageing. These are opposed by evolved genetic mechanisms for somatic repair, maintenance and longevity (Zimniak, 2012). In line with this view, the disposable soma theory has focused on ageing in the context of physiological trade-offs (Kirkwood, 2002). Costly somatic repair mechanisms counteract randomly accumulated molecular and cellular damage, and organisms must balance the investment of limited resources into such processes versus investment into germline maintenance and reproduction. The level of somatic maintenance, and therefore ageing, depends on the optimization of reproductive success for different environmental conditions.

A number of proximal, or mechanistic, explanations for senescence have been proposed. The free radical theory of ageing postulates that the accumulation of oxidative damage, through the production of reactive oxygen species (ROS) by mitochondrial respiration, underlies age-related degeneration (Harman, 1956). This theory, however, has largely given way to a realization that ROS are important signals for coordinating cellular stress responses (Ristow and Schmeisser, 2011), and that the location and quantity of ROS are key to their effects on the ageing process. In fact, a low level of stress may be beneficial and increase lifespan – a phenomenon referred to as 'hormesis' (Gems and Partridge, 2008; Yang and Hekimi, 2010). The induction of endogenous defence mechanisms that results from

these signals can entail enhanced stress resistance and longevity in model organisms (Lee *et al.*, 2010; Schulz *et al.*, 2007; Tauffenberger *et al.*, 2016). Moreover, a recent insight has emphasised the inappropriate continuation of growth-related developmental programs into adulthood, rather than untargeted molecular damage, as the primary driver of ageing (the 'hyperfunction' theory, Blagosklonny (2012)). This is an example of antagonistic pleiotropy, in which ageing is a 'quasi-program' that arises from chronic activation of intracellular signalling that evolved to orchestrate development. Hyperfunction theory offers a convincing alternative to disposable soma theory for understanding age-related degeneration and disease.

## 1.2 *Caenorhabditis elegans* as a model for understanding ageing and lifespan

### 1.2.1 *C. elegans:* life cycle and physiology

*Caenorhabditis elegans* (*C. elegans*) is a bacterivorous, free-living nematode found in temperate soils around the world. The mating system of *C. elegans* is androdioecious (males and hermaphrodites); the majority of the F1 progeny from a hermaphrodite self-fertilising develop into hermaphrodites, whilst a much smaller proportion (0.2%) become X0 males as a result of non-disjunction during meiosis I. At 20 °C, embryogenesis in *C. elegans* takes approximately 16 hours, and occurs in a highly resistant and impermeable egg shell that can withstand the bleaching protocols commonly used to obtain synchronized *C. elegans* populations. Post-embryonic development in *C. elegans* proceeds through four larval stages (L1-L4), separated by molts of the cuticle (Fig. 1. 1). *C. elegans* body size increases from approximately 0.25 mm after hatching to 1 mm in adults (Corsi *et al.*, 2015). The process of gonad development, or gonadogenesis, is initiated shortly after hatching and is completed in the final larval stage, L4. Hermaphrodites initially devote resources into the production of sperm, but germ line fate is switched to initiate the production of oocytes at the L4/adult transition (Corsi *et al.*, 2015).

At 20 °C, the entire life cycle from egg to adult takes around 3.5 days on a diet of *E. coli* OP50. However, *C. elegans* exhibits plasticity in its development, and can respond to stressful conditions including overcrowding, extremes in temperature or shortage of food by entering into reversible alternative larval stages. The first of these (L1 arrest) occurs at the L1 stage and is associated with enhanced stress resistance (Baugh, 2013). The primary signal for L1 arrest is food shortage,

and development will only proceed in the presence of sufficient food. As such, L1 arrest is considered to be a type of starvation-induced quiescence (Baugh, 2013). The second arrest, known as dauer diapause, occurs as an alternative third larval stage, and depends on a wider range of cues including temperature, low food levels and particularly overcrowding (as sensed by pheromone accumulation) (Fielenbach and Antebi, 2008).

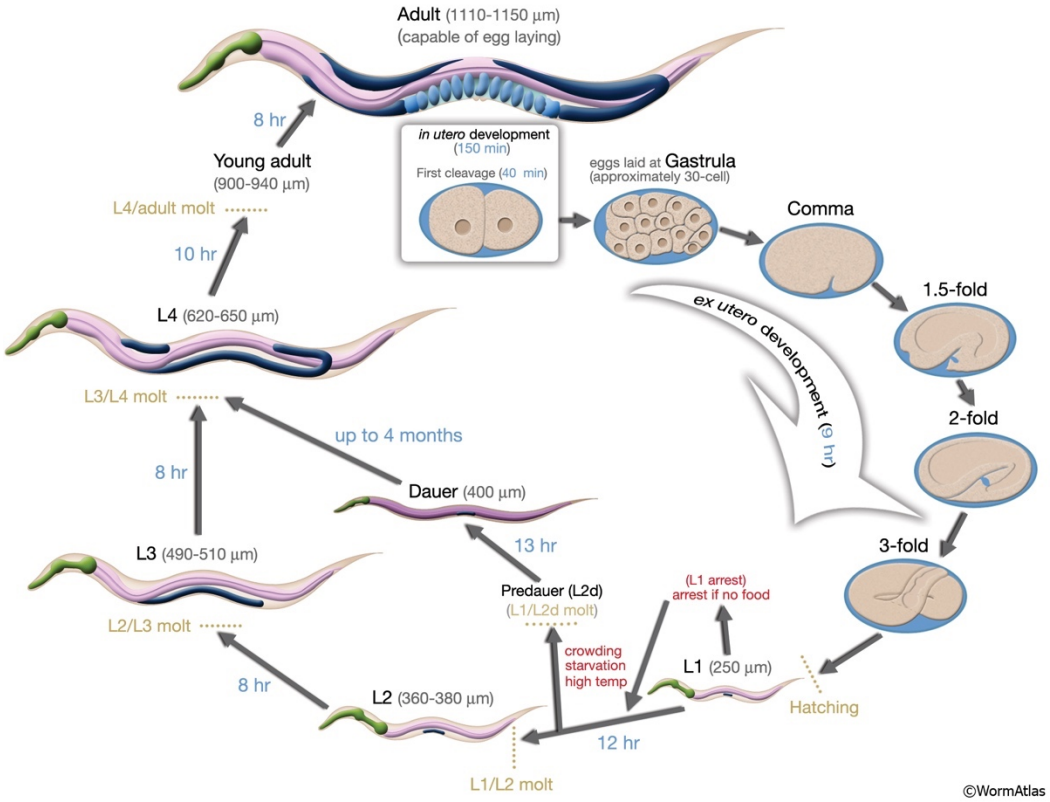



**Figure 1. 1 Life cycle and development of *C. elegans* hermaphrodites at 22 ° C.** Post-embryonic development of *C. elegans* proceeds through a series of larval stages, separated by cuticle molts. The dauer stage, an alternative L3 larval stage, is entered into under conditions of stress including food shortage, high temperature and overcrowding, and displays morphological and physiological adaptations for survival and stress resistance (Fielenbach and Antebi, 2008). *C. elegans* can also become arrested in the L1 stage of development under suboptimal conditions including heat stress and nutrient deprivation. *C. elegans* remains in L4, the final larval stage, for around 10 hours before molting into egg laying adults. Spermatogenesis (sperm production) begins during the L4 stage. Image reproduced from Altun and Hall (2018).

### 1.2.2 The utility of *C. elegans* in ageing research

*C. elegans* was first proposed as a promising model organism by Sydney Brenner (Brenner, 1974), and has since become one of the foremost model systems in biological research. *C. elegans* is a particularly popular model organism

for understanding the genetic and environmental factors influencing lifespan and ageing. This is largely owing to a number of characteristics including a short lifespan (2-3 weeks at 20°C), short life cycle, high fecundity, ease of propagation and well curated, readily available genetic resources including Wormbase (Stein *et al.*, 2001) and Caenorhabditis Genetics Center (CGC, University of Minnesota). Such features have facilitated large-scale and rapid screens for interventions influencing lifespan. Owing to the rarity of males and the advantages of a self-fertile system, hermaphrodites are the primary focus of the majority of *C. elegans* research.

One of the primary insights from *C. elegans* research is the deep level of conservation across animals in a variety of processes. This conservation has led to important discoveries, including the dissection of programmed cell death (Ellis *et al.*, 1991), microRNAs (Lee *et al.*, 1993; Reinhart *et al.*, 2000), and RNA interference (Fire *et al.*, 1998). The Nobel Prize committee recognized the implications of these discoveries for human health. Homology of signalling pathways extends to the processes that modulate ageing and lifespan; many genes that are deregulated in human ageing-associated diseases similarly regulate ageing in *C. elegans* (Torgovnick *et al.*, 2013). Senescence in *C. elegans* is characterised by many of the phenotypes associated with ageing in humans, such as wrinkling of the skin, muscle wasting, and decline in reproductive and cognitive capabilities (Bansal *et al.*, 2015; Herndon *et al.*, 2002; Murakami *et al.*, 2005). The motility of *C. elegans* shows an age-related decline, as does their capacity for chemotaxis and learning (Murakami *et al.*, 2005) and their resistance to bacterial infection (Garigan *et al.*, 2002; Garsin *et al.*, 2003).

Lifespan, defined as the period of time between birth and death, is frequently used as a convenient measure of ageing. Although extended lifespan is generally seen to reflect increased robustness and health with age, this connection has been frequently called into question. Studies using long-lived mutants of *C. elegans* show that, while overlapping, lifespan and ageing are not interchangeable terms (Tissenbaum, 2012; Tissenbaum, 2015). The focus in studies of ageing is therefore being shifted onto the so-called ‘healthspan’ of an organism, which is defined as the period of healthy lifespan before the onset of age-associated functional decline (Tissenbaum, 2012). In *C. elegans,* measures of healthspan have included changes in motility, pharyngeal pumping and stress resistance with age (Onken and Driscoll, 2010).

### 1.3 Ageing-associated pathways in *C. elegans*

A number of inter-connected genetic pathways that modulate the ageing process and influence lifespan and stress resistance have been described. At least five broad pathways – Insulin/IGF-1 signalling, mitochondrial signalling, germline signalling, neuronal signalling and pathways related to nutritional status and food quality – influence ageing through a set of independent transcription factors (Torgovnick *et al.*, 2013) (Fig. 1. 2). In some cases, changes in cellular homeostatic processes including autophagy and the mitochondrial unfolded protein response ($UPR^{mt}$) mediate these effects, acting as a point of convergence. The dissection of these pathways offers some promise for intervening in the process of ageing itself.

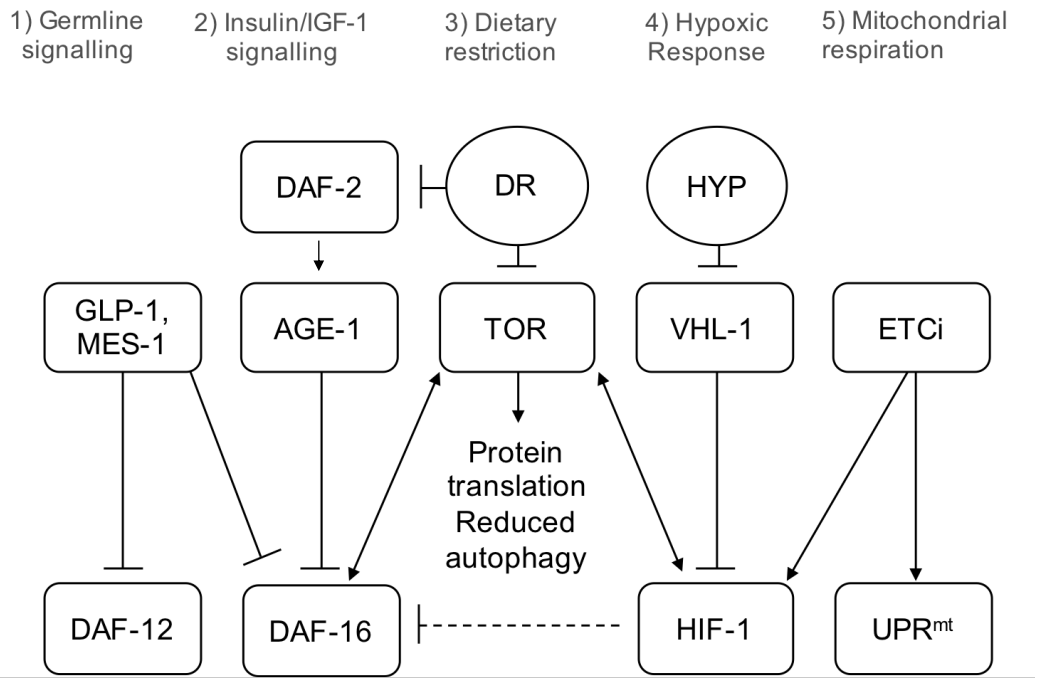



**Figure 1. 2 Simplified schematic of the signalling pathways that influence lifespan, ageing and stress resistance in *C. elegans*.** Genetic (rectangles) and environmental (circles) factors work through several major longevity pathways. 1) Mutation of germline signalling components (*mes-1, glp-1*) leads to longevity through DAF-16 and the putative hormone receptor DAF-12. 2) and 3) DR and IIS pathways converge on similar targets including DAF-16 and heat shock factor 1 (HSF-1). 4) Hypoxic conditions inhibit ubiquitination of hypoxia-inducible factor 1 (HIF-1) by VHL-1 and promote longevity. 5) Interfering with components of the mitochondrial electron transport chain (ETC inhibition, ETCi) also extends lifespan. These pathways can affect ageing through influencing homeostatic cellular mechanisms including protein translation, autophagy, energy metabolism and modulation of the $UPR^{mt}$. Figure modified from Yanos *et al.* (2012).

### 1.3.1 Insulin/IGF-1-like signalling in *C. elegans* and its relationship to ageing

The first ageing-associated pathway to be identified in *C. elegans* was the insulin/IGF-1-like signalling (IIS) pathway. Single gene mutations in this pathway, including the first *C. elegans* longevity gene, *age-1* (Friedman and Johnson, 1988) were demonstrated to have a dramatic influence on lifespan. Mutation of *daf-2* was subsequently found to elicit a two-fold extension of lifespan in *C. elegans* (Kenyon *et al.*, 1993). Strains of *C. elegans* with mutations in *daf-2* or *age-1* are long-lived and highly resistant to a range of stresses, including hypoxia, heat, heavy metals and bacterial pathogens (Kaletsky and Murphy, 2010). The longevity induced by both *daf-2* and *age-1* mutation was shown to be dependent on the gene *daf-16,* indicating that these components function in a common genetic pathway (Dorman *et al.*, 1995). The identification of single gene mutations that can dramatically influence longevity and health supports the view of ageing as a genetically regulated process (Herndon *et al.*, 2002).

The molecular function of these genes, and additional components, were subsequently characterised Fig. 1. 3. Signalling through the IIS is initiated by the binding of insulin-like peptide (ILP) ligands to the single insulin-like receptor, DAF-2, a homolog of the human insulin/IGF-1 receptor. Upon ligand binding, the receptor dimerises and residues in the cytoplasmic domain become auto-phosphorylated. The phosphatidylinositol 3-kinase (PI3K) AGE-1 (the catalytic subunit of which is encoded by *age-1*) is phosphorylated and activated by DAF-2 (Torgovnick *et al.*, 2013). AGE-1 phosphorylates phosphatidyl inositol-(4,5)-bisphosphate ($PIP_2$) to phosphatidyl inositol-(3,4,5)-trisphosphate ($PIP_3$) at the plasma membrane. $PIP_3$ in turn activates the kinase AKT-1 (ortholog to protein kinase B in mammals), which complexes with AKT-2 and SGK to phosphorylate and inactivate the Forkhead box O (FOXO) transcription factor DAF-16. This prevents its entry into the nucleus, where it promotes the expression of genes leading to longevity and stress resistance. Mutation of *age-1* or *daf-2* therefore relieves this inhibition and allows DAF-16 to enter the nucleus and activate genes associated with stress resistance, autophagy, protection from proteotoxicity, lipid metabolism and, ultimately, longevity (Kaletsky and Murphy, 2010). The IIS pathway also functions to integrate food and stress signals in developing *C. elegans* larvae to control entry into dauer diapause. When food is abundant, signalling through DAF-2 is increased, and DAF-16-mediated longevity and stress resistance phenotypes are suppressed (Kaletsky and Murphy, 2010), whilst reduced signalling promotes entry into dauer.

The target genes of DAF-16 are varied and include heat shock proteins (hsp's) and members of the superoxide dismutase (sod) family (Cohen *et al.*, 2006; Panowski *et al.*, 2007). Sod genes are central to oxidative stress resistance in *C. elegans,* and the best characterised of these is *sod-3,* a mitochondrial Fe/Mn superoxide dismutase. The expression of *sod-3* is specifically elevated in response to a reduction in IIS signalling (Panowski *et al.*, 2007). The heat shock factor 1 (HSF-1) is also negatively regulated by IIS signalling, and a number of the target genes of HSF-1 overlap with those of DAF-16. HSF-1 is a central component of the heat shock response (HSR) and its activation is involved in enhancing stress resistance in *C. elegans* (Maman *et al.*, 2013).

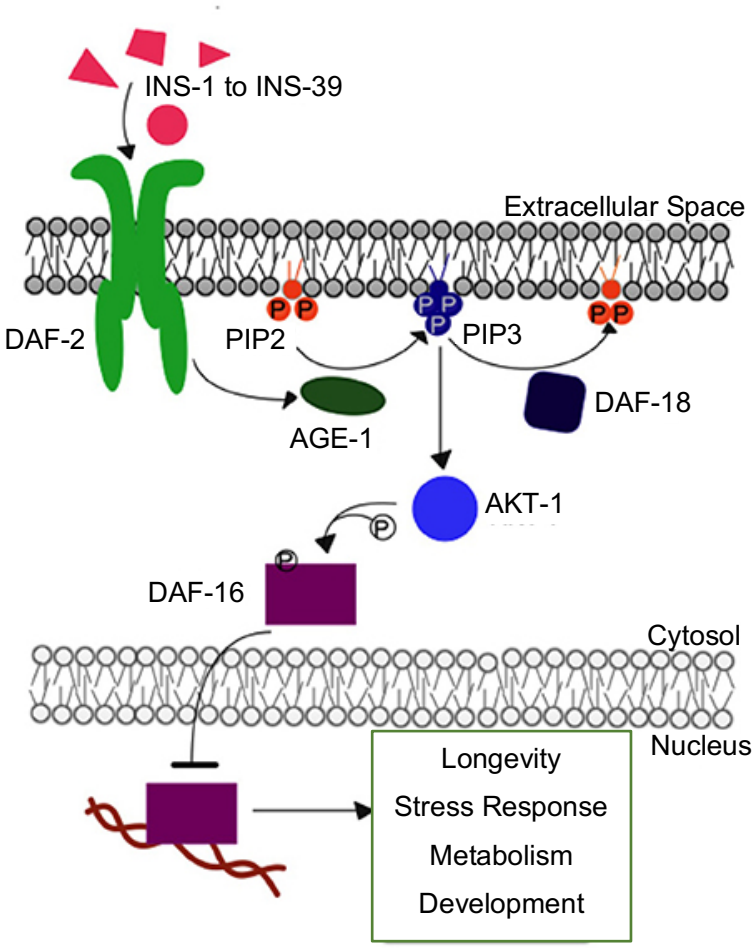



**Figure 1. 3 Insulin/IGF1-like signalling (IIS) in *C. elegans*.** See main text for details. Figure reproduced from Scerbak *et al.* (2014).

The IIS signalling pathway is highly evolutionarily conserved, and functions to modulate lifespan and ageing in organisms from *D. melanogaster* to mammals. In humans, variants of IIS pathway components have been linked to longevity (Pawlikowska *et al.*, 2009). DAF-16 has homology to human FOXO transcription factors, and genetic variation in one of these (FOXO3a) is strongly associated with human longevity (Willcox *et al.*, 2008).

### 1.3.2 Dietary restriction and lifespan in *C. elegans*

The primary non-genetic intervention that is capable of robustly extending lifespan and improving health is dietary restriction (DR), defined as a reduction in food intake below *ad libitum* levels, but without malnutrition. As well as a general restriction in macronutrients, DR can encompass more specific changes in the levels of micronutrients and shifts in the pattern of feeding (Lee and Longo, 2016). DR elicits beneficial effects in a wide range of different species, including yeast, nematodes, flies, rodents and primates (Lee and Longo, 2016). DR also has potential as an anti-ageing intervention in humans; DR is capable of reducing body fat and insulin levels, lowering body temperature (Redman and Ravussin, 2011) and reducing ageing-associated biomarkers in humans (Most *et al.*, 2017). As well as longevity, DR can be associated with some characteristic changes in morphology and physiology. Nematodes which undergo DR share a set of common phenotypes, including small body size, delayed development and reduced fecundity, as well as enhanced resistance to heat and oxidative stress (Houthoofd *et al.*, 2002). These animals also display metabolic changes including increased autophagy as indicated by higher numbers of autophagosomes within seam cells, a reduction in triglyceride levels and decreased fat staining (Heestand *et al.*, 2013).

There are a number of theories as to the mechanisms behind DR-mediated longevity. DR has been suggested to arise through reduced ROS generation, consistent with the free radical theory of ageing (Harman, 1956), or by a reduction in metabolic rate and an entailing reduction in ROS. However, there are indications from several model species including yeast and *C. elegans,* that metabolic rate is actually increased by DR (Houthoofd *et al.*, 2005). Alternatively, the benefits of DR might arise from an upregulation of stress defence that in turn extends lifespan and enhances stress resistance (Zhou *et al.*, 2011) (i.e. a hormetic mechanism). In line with the disposable soma theory of ageing, DR has also been suggested to extend lifespan by diverting resources from reproduction to somatic maintenance

(Kirkwood, 2005). Reduced IIS and target of rapamycin (TOR) signalling mediate a number of the effects of DR on lifespan and health (discussed further in Chapter 4).

### 1.3.3 *C. elegans* stress responses and their relationship to ageing

*C. elegans* may be subject to various forms of stress throughout its lifespan, including temperature extremes, overcrowding and food shortages. At the cellular level, such stresses entail the induction of heat shock, oxidative stress, hypoxia and osmotic stress responses (Rodriguez *et al.*, 2013). The neuroendocrine pathways that these responses encompass include the IIS and transforming growth factor-β (TGF-β) pathways, through downstream effectors including DAF-16 and HSF-1 in the case of the HSR (Rodriguez *et al.*, 2013). Cellular stress can also arise through the generation of ROS, primarily by mitochondria, as a by-product of oxidative phosphorylation. Oxidative stress can disrupt lipids, DNA and proteostasis, and must therefore be neutralized. The $UPR^{mt}$, characterised by the activity of molecular chaperones including HSP-6 and HSP-60, operates as one branch of the response to cellular stress to maintain mitochondrial protein homeostasis in the face of ROS-induced damage (Tauffenberger *et al.*, 2016). The $UPR^{mt}$ is activated in *C. elegans* through electron transport chain disruption, mitonuclear protein imbalance or disruption of mitochondrial protein homeostasis (Cai *et al.*, 2017).

Although longevity appears to be commonly associated with increased resistance to stress, whether stress resistance is causal to longevity is a matter of debate. The accumulation of stress-induced damage has been viewed as central to the ageing process. A number of studies in different model systems have indicated that longevity is connected to reduced oxidative stress (Ristow and Schmeisser, 2011). A delay in ageing might therefore arise through an enhanced ability to cope with molecular damage (Gems and Partridge, 2008). Seemingly in support of this view, the overexpression of heat shock proteins or HSF-1 can extend lifespan and improve stress resistance in *C. elegans* (Hsu *et al.*, 2003).

The modulation of the IIS pathway in *C. elegans* has revealed an association between longevity, stress resistance and proteostasis. This association is apparent not just in nematodes, but also in flies and mice (Gems and Partridge, 2008). *C. elegans* mutants of IIS are more resistant to external stressors and are less susceptible to aggregation of the Aβ1-42 peptide in *C. elegans* models Alzheimer's-related proteotoxicity (Cohen *et al.*, 2006). However, the downstream

functions of the IIS can in many cases be decoupled from the effect of the pathway on longevity and ageing. For example, deleting all five of the superoxide dismutase (sod) genes in *C. elegans* did not affect lifespan, but the nematodes became hypersensitive to oxidative stress (Van Raamsdonk and Hekimi, 2012). This also demonstrates that a normal rate of ageing (as reflected in lifespan) does not necessarily require the maintenance of stress resistance and calls into question the oxidative damage theory of ageing, as had a previous study focusing on the role of the superoxide anion in *C. elegans* ageing (Doonan *et al.*, 2008). Moreover, the extended lifespan of *C. elegans* in which *sod-1* is overexpressed was found to result from activation of longevity-promoting transcription factors including DAF-16, rather than from a reduction in the levels of superoxide free radicals (Cabreiro *et al.*, 2011). Similarly, knock-down of a G-protein coupled receptor that is required for the activation of the HSR protected *C. elegans* from proteotoxicity but did not affect lifespan (Maman *et al.*, 2013). Therefore, at least under certain circumstances, longevity can be decoupled from improved stress resistance.

The phenomenon of hormesis has also received experimental support from studies in *C. elegans.* An elevation of ROS or sublethal doses of oxidants can induce lifespan extension and promote stress resistance in *C. elegans,* in processes that are mediated by transcription factors including DAF-16, SKN-1 and HIF-1 (Lee *et al.*, 2010; Schmeisser *et al.*, 2013; Yang and Hekimi, 2010).

## 1.4 Diet, microbiota and ageing

### 1.4.1 The intestinal microbiota

The characteristic microbial community occupying a particular environment is referred to as the microbiota. In mammals, the microbiome – defined as the genes, genomes and products of the microbiota and the host habitat it occupies (Whiteside *et al.*, 2015) – is central to host physiology. The gut microbiota has received particular attention, due to its profound role in functions ranging from digestion, education of the immune system and protection against pathogens (Lee and Hase, 2014; Shreiner *et al.*, 2015).

The intestine represents a central interface between host and microbiome. In humans, the gastrointestinal (GI) tract is home to the highest number and concentration of microbes in the body (Saraswati and Sitaraman, 2015). Various

disease states in humans, including chronic GI diseases (ulcerative colitis, Crohn's disease), metabolic disorders (obesity, type 1 and type 2 diabetes), asthma and arthritis, have been linked to dysregulation of the microbiota (dysbiosis) as well as changes in the relative quantity of dietary components (Richards *et al.*, 2016; Saraswati and Sitaraman, 2015). Dysbiotic states are characterised by inherent variability, making the identification of a consistent marker challenging (Clark and Walker, 2018). The production of short-chain fatty acids by the gut microbiota during fermentation of fibre has been linked to beneficial anti-inflammatory and homeostatic effects (Richards *et al.*, 2016). Therefore, the concept of changing dietary intake or shifting the microbial composition of the gut to alter disease susceptibility has received increasing attention (Richards *et al.*, 2016).

Dysbiosis and dietary intake are closely connected, as the microbial ecology of the gut is shaped by diet. Broadly, the high-fat, protein-rich diet typical in Western societies has been associated with a microbiome dominated by *Bacteroides,* whilst a high-fibre diet is linked to a Firmicutes- and Proteobacteria-dominated ecology (Wu *et al.*, 2011). In contrast, the *Prevotella* enterotype was strongly associated with a long-term carbohydrate-rich diet. Age-related shifts in microbial composition are also apparent, with a higher proportion of Bacteroidetes present in ageing individuals. In one study, individuals with higher frailty scores had an increase in the proportion of *Atopobium* and Enterobacteriaceae, and a decrease in the proportion of other groups including *Bacteroides* and lactobacilli, linking microbial diversity with the processes of age-related decline (van Tongeren *et al.*, 2005). The host and the microbiota reciprocally influence one another, such that cause and effect are difficult to discern. Moreover, there exist distinct ecological niches along the human alimentary canal, complicating the correlation of microbial composition with healthy or diseased states (Saraswati and Sitaraman, 2015). Identification of the molecules mediating host-microbiota interactions and how these processes are regulated is therefore particularly challenging.

### 1.4.2 The interplay between microbial activity, nutrition and nematode ageing

*C. elegans* provides a simple and effective system in which to study the complex interactions between diet and microbiota, as it is usually kept on a bacterial monoculture of the mammalian gut bacterium *E. coli* that has undergone decades of adaptation to the laboratory environment (Brenner, 1974). Simply substituting a different bacterial monoculture can enable investigation into the impact of species-

specific nutrients on lifespan, ageing and other aspects of *C. elegans* biology (MacNeil *et al.*, 2013; Sánchez-Blanco *et al.*, 2016). The intestine of *C. elegans* is composed of only 20 cells and performs both digestive and endocrine functions (MacNeil *et al.*, 2013). Moreover, the consumption of live bacteria is important for *C. elegans* health and lifespan, as a diet of *E. coli* rendered metabolically inactive with β-irradiation fails to rescue the effects of DR (Lenaerts *et al.*, 2008). Whilst most of the bacterial cells that *C. elegans* ingests are broken down by the pharyngeal grinder to be used as a food source, some escape and remain alive inside the *C. elegans* gut (Garigan *et al.*, 2002; Portal-Celhay and Blaser, 2012). Since the bacterial diet directly and indirectly influences micronutrient supply of *C. elegans*, it has been likened to the gut microbiota in higher organisms (Heintz and Mair, 2014; Watson *et al.*, 2014).

### 1.4.3 The *C. elegans* microbiome

In the laboratory, *C. elegans* is usually maintained on a monoxenic culture of *E. coli*. The descendants of two *E. coli* isolates lacking the oligosaccharide O-antigen - strain K12 and B strain – are common food sources, in particular the B strain derivative *E. coli* OP50 (Brenner, 1974). This strain, a uracil auxotroph, is translucent, sticky and slow-growing which makes it well-suited for the purpose of nematode maintenance. *E. coli* is commonly found within the intestine of endotherms and is not likely to be commonly encountered by *C. elegans* in its natural environment. Moreover, the standard laboratory strain of *C. elegans,* N2, is genetically distinct from wild *C. elegans* strain as a result of laboratory domestication (Sterken *et al.*, 2015). As such, whilst this system has provided many great insights into diverse areas of biology, its simplicity masks the aspects of *C. elegans* biology which require the presence of more varied interacting species (Samuel *et al.*, 2016). The details of the natural ecology of *C. elegans* in terms of its food source, predator-prey interactions are response to natural environmental challenges are only starting to be uncovered (Frézal and Félix, 2015; Samuel *et al.*, 2016).

In its natural environment, *C. elegans* feeds on a variety of bacterial species that live on decomposing vegetation (Dirksen *et al.*, 2016; Félix and Duveau, 2012; Samuel *et al.*, 2016). Several recent studies have shed some light on the microbial community which inhabits and interacts with *C. elegans* in its natural habitat (Berg *et al.*, 2016; Dirksen *et al.*, 2016; Samuel *et al.*, 2016). 16S ribosomal DNA deep

sequencing revealed that the dominant bacterial taxa belong to Proteobacteria, with Actinobacteria and Firmicutes also represented (Dirksen *et al.*, 2016). Interestingly, when germ free L1 larvae were fed on soil with different microbial compositions, the adult microbiotas from each environment resembled one other (Berg *et al.*, 2016). This reveals that the host tunes the composition of the microbiome.

### 1.4.4 Alteration of *C. elegans* development and ageing by bacterial signals

The availability of the bacterial food source and the amount of nutrition it provides are key factors affecting nematode physiology and lifespan (Watson *et al.*, 2014), (Avery and Shtonda, 2003). Changes in bacterial diet are able to modulate *C. elegans* life history traits, including development, fecundity, lifespan and stress resistance (Coolon *et al.*, 2009; Kim and Mylonakis, 2012; MacNeil *et al.*, 2013; Shtonda and Avery, 2006). Many of these effects are likely to be mediated by differences in the composition of macronutrients, such as carbohydrates, proteins and fats. However, the presence or absence of particular bacterially-derived micronutrients – including vitamins, cofactors and minerals – can also greatly influence the physiology of *C. elegans* (Yilmaz and Walhout, 2014). As well as simply changing the nutritional content of the diet, these factors are able to activate host signalling pathways to modulate lifespan and health.

Notably, in one study the soil bacterium *Comamonas,* which has been isolated from samples containing *C. elegans* (Avery and Shtonda, 2003), accelerated development and reduced the fecundity and lifespan of *C. elegans* compared to those fed *E. coli* OP50 (MacNeil *et al.*, 2013). Shifts in gene expression accompanied these changes, including repression of the acyl-CoA dehydrogenase-encoding gene *acdh-1*. The authors developed this gene into a dietary sensor (*Pacdh-1::gfp*) to monitor the response of *C. elegans* to *Comamonas.* Surprisingly, dramatically diluting *Comamonas aquatica* DA1877 in *E. coli* OP50 increased developmental rate in *C. elegans* and repressed the dietary sensor*,* demonstrating that nematode physiology is altered by a signal produced by the bacteria, rather than as a consequence of differences in macronutrient composition. This signal affects the molting program of *C. elegans*, mediated by the nuclear hormone receptor NHR-23, in order to influence developmental rate. The nature of the primary dilutable signal involved in the response to *Comamonas* was identified as vitamin B12, which is not produced by *E. coli* OP50 (Watson *et al.*, 2014).

Supplementation of vitamin B12 recapitulated the accelerated developmental rate and reduced fertility of *C. elegans.*

As well as vitamin B12, another micronutrient which strikingly affects *C. elegans* biology is vitamin B9, or folate. *C. elegans* cannot synthesise folate and must instead obtain it from its diet. Bacterial folates stimulate germ-cell proliferation in *C. elegans* hermaphrodites through a pathway that is dependent on the folate receptor homolog FOLR-1 (Chaudhari *et al.*, 2016). Moreover, interfering with folate synthesis in *E. coli,* either chemically or through mutation of *aroD,* is capable of extending the lifespan of *C. elegans* by over 30% (Virk *et al.*, 2012). This did not arise as a consequence of changes in *C. elegans* folate levels*,* but instead through further metabolic shifts in *E. coli,* which entailed the suppression of mildly toxic bacterial activities (Virk *et al.*, 2016).

Recently, the genetic resources of *E. coli* were utilised to conduct a genome-wide screen for *E. coli* genes that influence lifespan in *C. elegans.* Heightened production of the polysaccharide colanic acid (CA) by certain *E. coli* mutants was shown to promote longevity and health in *C. elegans* following its uptake into intestinal cells by endocytosis (Gruber and Kennedy, 2017; Han *et al.*, 2017). CA had similar beneficial effects in *Drosophila* and mammalian cell lines. CA is produced in response to bacterial stress and extends lifespan in *C. elegans* via the induction of mitochondrial fragmentation and the $UPR^{mt}$ in intestinal cells. This study therefore provides a fascinating link between microbial stress and the induction of the stress response in *C. elegans,* indicating that *C. elegans* might use markers of bacterial stress to prepare for stressful conditions.

Changes in bacterial metabolism can also alter the effect of certain drugs on the physiology of *C. elegans.* The biguanide drug metformin is administered as treatment for Type 2 diabetes and is the world's most widely prescribed drug (Cabreiro *et al.*, 2013). However, this drug also offers exciting promise as an anti-ageing intervention, as it can delay ageing in rodents and *C. elegans* (Onken and Driscoll, 2010). A study in *C. elegans* found that metformin recapitulated many of the effects of DR in an AMP-activated protein kinase (AMPK) and SKN-1-dependent and IIS-independent manner (Onken and Driscoll, 2010). This included an extension of median lifespan, reduced body fat and reduced fecundity. Interestingly, metformin actually reduced the lifespan of *C. elegans* in axenic culture, indicating that bacteria play a key role in mediating the effect of the drug (Cabreiro *et al.*,

2013). Nematode lifespan was also reduced by metformin in the presence of certain *E. coli* strains, and this was more pronounced when the bacteria were less sensitive to metformin-induced growth inhibition. However, rather than limiting growth *per se* to increase *C. elegans* lifespan, the authors found that metformin altered folate and methionine metabolism in *E. coli,* leading to a state of methionine-restriction in *C. elegans* that elicited longevity and health through DR pathways. A subsequent study showed that this health improvement requires lysosome-dependent activation of AMPK (Chen *et al.*, 2017). This demonstrates the utility of *C. elegans* as a model system for uncovering novel and unexpected links between microbiota and drug therapy.

The effect of diet on *C. elegans* ageing is dynamic and regulated by adaptive host mechanisms. Fascinatingly, the mutation of a single gene, *alh-6,* in *C. elegans* results in rapid ageing on a diet of the *E. coli* B strain derivative OP50, but not the K12 derivative *E. coli* HT115, as a consequence of diet-dependent shifts in host proline catabolism (Pang and Curran, 2014). A diet of *E. coli* HT115 prevents sterility in *C. elegans* with a mutation of nuclear hormone receptor-114 (NHR-114), as a consequence of the status of tryptophan metabolism in this strain (Gracida and Eckmann, 2013).

### 1.4.5 Respiration-deficient bacterial diets

One set of metabolic mutants that have a striking influence on the lifespan of *C. elegans* are those that are deficient in respiratory metabolism. When *C. elegans* is fed on *E. coli* lacking the antioxidant electron carrier coenzyme Q (coQ), which functions in the mitochondrial respiratory chain, its lifespan is extended (Gomez *et al.*, 2012). Similarly, two *E. coli* mutants for ATP synthase, *E. coli* AN120 (harbouring a mutation in the alpha subunit of ATP synthase) and *E. coli* 1100 Δbc (lacking the entire ATP synthase operon) have life-extending effects. When fed on 1100 Δbc, the lifespan of *C. elegans* increases by around 19% compared to growth on the parent strain 1100, in the absence of glucose (Saiki *et al.*, 2008).

Respiration-deficient strains of *E. coli* have reduced growth rate in liquid and on solid media (Gomez *et al.*, 2012; Santana *et al.*, 1994). One possibility is that the probiotic effect of these diets arises from their effect on the growth rate of *E. coli.* This hypothesis is based on the realization that *E. coli* exerts some mildly pathogenic effects on *C. elegans,* as suggested by several lines of evidence. For

example, studies have frequently reported that *C. elegans* live longer on dead rather than live lawns of *E. coli* OP50 (Garigan *et al.*, 2002; Gems and Riddle, 2000). Moreover, feeding *C. elegans* on live bacteria whose growth had been arrested by carbenicillin also increased *C. elegans* lifespan, indicating that growth and proliferation of *E. coli* rather than the presence of live bacteria *per se* is harmful (Garigan *et al.*, 2002). The potentially harmful aspect of *E. coli* growth that has received considerable attention is intestinal colonisation in ageing nematodes. Convincing links have been made between proliferation of *E. coli* within the intestine of *C. elegans,* and morbidity and premature death (Browning *et al.*, 2013; Garigan *et al.*, 2002; Gomez *et al.*, 2012; Kim, 2013; Podshivalova *et al.*, 2017). Gomez *et al.* (2012) used GFP-tagged *E. coli* lacking coQ or ATP synthase in order to monitor the level of gut colonization, and also took direct measures of colony forming units. The authors determined that increased levels of excreted compounds, including D-lactic acid, were not responsible for life span extension. Instead, they found a correlation between increased intestinal load and reduced lifespan, with respiration-deficient strains less able to colonise *C. elegans.* These animals, it is proposed, undergo less stress and live longer as a result. Similarly, in another study the number of viable bacteria in the *C. elegans* intestine was strongly correlated with lifespan (Portal-Celhay *et al.*, 2012).

However, whether over-proliferation of *E. coli* in the nematode intestine is causal to ageing is a matter of considerable debate. One report (Hsiao *et al.*, 2013) determined that the level of fluorescence in GFP-tagged bacteria was the same whether the bacteria were alive or dead, and so cannot be used as to estimate bacterial colonization (as in Gomez *et al.* (2012)). In another study (Virk *et al.*, 2016), in which individuals rather than populations (as in Portal-Celhay *et al.* (2012)) were assessed, around half of recently dead *C. elegans* did not display any visible intestinal accumulation of *E. coli.* The authors suggest that gut accumulation may represent a good biomarker, rather than a cause, of aging – a decline in intestinal integrity, pharyngeal pumping efficiency and defecation would likely promote bacterial accumulation in ageing individuals (Virk *et al.*, 2016). Instead, they propose, the pathogenicity of *E. coli* might arise from the combined effect of milder (and as yet unidentified) toxic activities on the host.

In support of this view, coQ itself has a negative effect on the lifespan of *C. elegans* (Sánchez-Blanco *et al.*, 2016). Supplementation of *B. subtilis*-fed nematodes with coQ-active *E. coli* extract reduced their lifespan more than the

addition of extract lacking coQ. This offers an explanation for the longevity of *C. elegans* fed on coQ-less *E. coli* which is distinct from a reduced capacity to colonise the nematode intestine. This example highlights that, as opposed to a common underlying mechanism of reduced gut colonisation, there may be factors specific to each respiration-deficient mutant that extend *C. elegans* lifespan.

Rather than bacterial load in the gut *per se,* it is likely that other toxic activities of *E. coli* produce mild pathogenic effects which might be exacerbated by prolonged proximity to intestinal cells. Supporting this, the increased intestinal load of non-pathogenic bacteria seen in *C. elegans* with impaired pharyngeal grinding only compromises lifespan when the immune system is impaired (Portal-Celhay *et al.*, 2012). Moreover, certain bacteria exhibit heavy gut colonisation with little effect on nematode lifespan (Garsin *et al.*, 2001).

### 1.4.6 Probiotic and protective effects of the microbiota

Many beneficial host-microbiome interactions have arisen as a consequence of intimate ecological associations; species of bacteria including *Gluconobacter* sp. and *Enterobacter* sp., that were isolated from the natural environment of *C. elegans* enhanced the resistance of the nematode to infection by species, including *Pseudomonas* sp., and *Chryseobacterium* sp., that are resident to the same habitat (Samuel *et al.*, 2016). *Pseudomonas mendocina,* another member of the *C. elegans* microbiome, enhances nematode resistance to *P. aeruginosa* infection (Montalvo-Katz *et al.*, 2013) in a PMK-1-(p38 MAPK)-dependent manner.

A range of bacterial species have begun to fall into the category of “probiotics”, defined as “live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host” (Hill *et al.*, 2014). Examples of this are the lactic acid bacteria (LAB), members of which are used as probiotics in the feed industry as well as in the treatment of inflammatory bowel diseases (Saez-Lara *et al.*, 2015). Fascinatingly, these bacteria also exert a probiotic effect in *C. elegans.* One study found that 24 hr incubation with the gram-positive bacterium *Lactobacillus acidophilus* NCFM enhanced resistance of *C. elegans* to pathogenic strains including *Staphylococcus aureus* and the human gut commensal *Enterococcus faecalis,* which required upregulation of specific immune response pathways (Kim and Mylonakis, 2012). Another study screened *C. elegans* against a set of LAB strains, and identified a strain capable of increasing nematode lifespan

and eliciting an anti-inflammatory effect in cell lines (Grompone *et al.*, 2012). Conversely, several strains of LAB are detrimental to *C. elegans* and cause the accumulation of lipid droplets as well as increased expression of genes previously linked to obesity phenotypes in *C. elegans* (including *pept-1, nhr-49*, and *tub-1*) (Zanni *et al.*, 2015).

Another group of bacteria which have received attention as potential probiotic interventions are *Bacillus* species. Unlike LAB, *Bacillus* species are capable of forming spores, which entails better survival in the acidic conditions of the stomach and higher stability during processing in food products (Bader *et al.*, 2012). Crucially, *Bacillus* species exhibit anti-oxidant, immune-modulatory, pathogen exclusion and food fermentation properties (Elshaghabee *et al.*, 2017). One species in particular, the rod-shaped, gram-positive bacterium *Bacillus subtilis,* fulfils many of the criteria for a probiotic intervention. Strains of *B. subtilis* are able to stimulate the immune system of elderly patients (Lefevre *et al.*, 2015) and have been used in the animal feed industry as a probiotic (Cutting, 2011).

### 1.5 *Bacillus subtilis* as a diet for *C. elegans*

#### 1.5.1 The effect of *B. subtilis* on *C. elegans* lifespan, health and stress resistance

*B. subtilis* is an extremely well characterized model organism. The highly transformable strain 168, a tryptophan-requiring auxotroph (*trpc2*), has been the subject of extensive genetic study for decades (Zeigler *et al.*, 2008). Strain 168 was selected for genome sequencing (Kunst *et al.*, 1997) and is therefore well established as a model bacterial species. It also benefits from the existence of a centralized mutant repository, the Bacillus Genetic Stock Center (BGSC).

*Bacilli* reside in the gastrointestinal tract of animals and are also present abundantly in the soil. Members of the *Bacillaceae* family are likely to comprise part of the natural diet of *C. elegans* (Berg *et al.*, 2016; Grewal, 1991), and form an association with this nematode under laboratory settings, in conjunction with a range of other species (Berg *et al.*, 2016; Dirksen *et al.*, 2016). *C. elegans* displays a preference for soil bacteria such as *B. mycoides* and *B. soli* over *E. coli* OP50 (Abada *et al.*, 2009). *Bacilli* have been identified as a component of the ‘core microbiome’ of *C. elegans,* which assembles from different microbial communities in a laboratory setting (Berg *et al.*, 2016) and positively influence the physiology of this

nematode. In fact, when grown on *Bacillus subtilis*, *C. elegans* does not seem to suffer from pathogenic effects associated with intestinal colonization and lives longer than in the presence of *E. coli* (Garsin *et al.*, 2003; Gusarov *et al.*, 2013). Dietary *Bacillus subtilis* is capable of increasing the mean lifespan of *C. elegans* in comparison to nematodes fed on *E. coli* from around 17 to 27 days (Garsin *et al.*, 2003; Gusarov *et al.*, 2013; Sánchez-Blanco and Kim, 2011). Similarly, *C. elegans* fed on *Bacillus megaterium* lived longer than those fed on *E. coli* (Coolon *et al.*, 2009) and exhibited resistance to *Pseudomonas aeruginosa* infection (Montalvo-Katz *et al.*, 2013). Interestingly, *B. megaterium* was isolated from the microbiota of *C. elegans* that was acquired in a natural-like soil environment (Montalvo-Katz *et al.*, 2013). Moreover, one strain of *B. subtilis,* GS67, was able to enhance the resistance of *C. elegans* to infection by Gram-positive bacterial pathogens, through the release of the lipopeptide fengycin which acted as an antibiotic (Iatsenko *et al.*, 2014).

### 1.5.2 Mechanisms by which *B. subtilis* promotes health and longevity in *C. elegans*

**Release of pro-longevity molecules**. Recently, our understanding of the mechanisms underlying these health benefits has been improved. Importantly, *E. coli* and *B. subtilis* have similar caloric content and macronutrient composition (Sánchez-Blanco *et al.*, 2016), indicating that differences in lifespan are not conferred by differences in nutrition. Instead, detrimental activities of *E. coli,* including intestinal proliferation, might mediate these differences. Indeed, it has been suggested that the lack of the antioxidant coQ production by *B. subtilis* is the primary reason for its pro-longevity effect, compared to a diet of *E. coli* (Sánchez-Blanco *et al.*, 2016). In particular, *C. elegans* fed on coQ-producing *E. coli* have a lower oxidation state, and altered cellular REDOX homeostasis is proposed to reduce lifespan in these animals. *E. coli* also produces components besides coQ, including lipopolysaccharides, that are detrimental to *C. elegans* (Maier *et al.*, 2010).

The relative health of *C. elegans* fed on *B. subtilis* is not, however, merely a consequence of reduced bacterial pathogenicity or toxic activity. Instead, factors produced by *B. subtilis* have an actively beneficial impact on *C. elegans.* The release of nitric oxide (NO) appears to mediate many of the beneficial effects of *B. subtilis* on *C. elegans. C. elegans* incorporates NO produced by other organisms

including *B. subtilis* (Donato *et al.*, 2017; Gusarov *et al.*, 2013), as it lacks nitric oxide synthases (NOS). Bacterially derived NO extends longevity and enhances stress resistance in *C. elegans* through DAF-16 and HSF-1-mediated pathways (Gusarov *et al.*, 2013). *B. subtilis* also releases the pentapeptide Competence Sporulation stimulating Factor (CSF), one of a number of effector molecules that enable bacterial communication and promote population survival and adaptation (a process known as quorum sensing (Fujiya *et al.*, 2007)). Exogenous addition of CSF to NGM plates increases the lifespan of *C. elegans* fed on *E. coli* OP50 (Donato *et al.*, 2017). The contribution of CSF release to the probiotic effects of *B. subtilis* has been recognized for some time; studies using human colonic epithelial cell lines demonstrated an activation of cytoprotective and survival pathways in response to CSF uptake through membrane transporters (Fujiya *et al.*, 2007).

**Biofilm formation.** As well as existing as free-floating, planktonic cells, microorganisms can assemble into structured assemblies, known as biofilms, which are encased in an extra-cellular matrix largely composed of proteins and polysaccharides. Within these populations, cells communicate and distribute nutrients via networks of channels (Donato *et al.*, 2017; van Gestel *et al.*, 2015), exhibiting coordinated social behaviours similar to that of multicellular organisms (Parsek and Greenberg, 2005). *B. subtilis* 168 has become domesticated following its culture under artificial conditions for many generations. During this process, it has lost many traits characteristic of wild strains, including architecturally complex biofilm formation (Fujiya *et al.*, 2007).

The proficiency of biofilm formation has been highlighted as a major factor in the ability of *B. subtilis* to promote healthy aging, including stress resistance and longevity. Undomesticated strains of *B. subtilis* increase stress resistance and prolong *C. elegans* lifespan to a greater extent than domesticated strains, and this depends on the formation of biofilms (Donato *et al.*, 2017). Although these authors found that a higher capacity for gut colonization was correlated with greater *C. elegans* lifespan, a subsequent study found no biofilm-dependent difference in the load of *B. subtilis* within the nematode intestine (Smolentseva *et al.*, 2017). Instead, changes in gene expression in the host mediate the beneficial effects of the biofilm on host physiology; for example, activity of *ilys-2* in *C. elegans,* which encodes a lysozyme involved in inmate immunity, underlies biofilm-mediated resistance to *P. aeruginosa* infection. Similarly, biofilm formation enhanced the resistance of *C. elegans* to oxidative stress and heat stress*,* mediated by the metallothionein Mtl-1

and the molecular chaperone HSP70, respectively (Smolentseva *et al.*, 2017). Although the production of NO and CSF is enhanced in *B. subtilis* growing as a biofilm (Donato *et al.*, 2017), these molecules seem to increase nematode lifespan independently of biofilm formation (Smolentseva *et al.*, 2017). The metabolites that are specific to biofilms and which induce longevity and stress resistance in *C. elegans* remain to be elucidated.

**Spore formation.** Other mechanisms for *B. subtilis*-induced longevity have received some attention. During vegetative growth, *B. subtilis* produces two identical cells through medial division (Tan and Ramamurthi, 2014). However, when nutrients become limiting, *B. subtilis* is able to form resilient and metabolically-inactive cells known as endospores (or spores) which can then germinate when conditions become more favourable for growth. At the onset of sporulation, cell division becomes asymmetric, producing a mother cell and a smaller pre-spore (Feucht *et al.*, 1996). The endospore is nurtured within the mother cell, which must lyse in order to release the spore (McKenney *et al.*, 2013). When environmental conditions improve, the spores germinate and resume vegetative growth Fig. 1. 4.

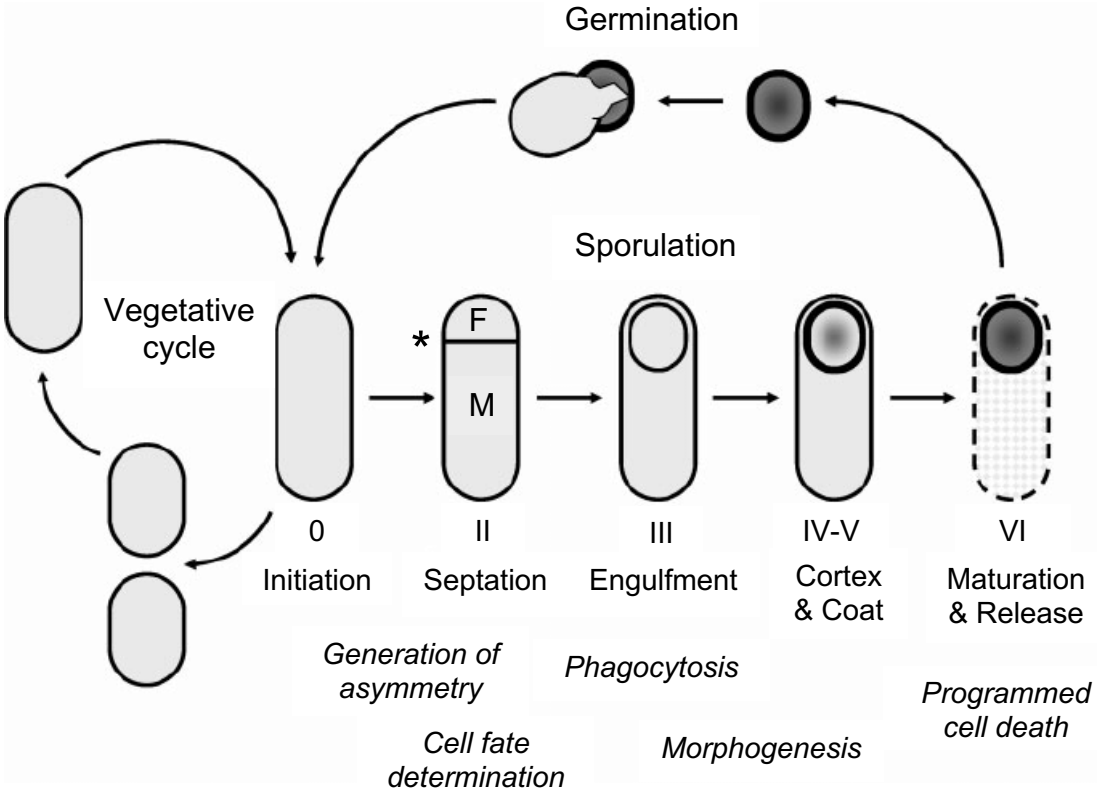



**Figure 1. 4 Sporulation and germination in *Bacillus subtilis.*** The first stage of sporulation is the asymmetric division of the sporangium to produce the forespore ('F') and the mother cell ('M'). These two compartments are separated by a septum. In the next stage, the forespore is engulfed by the mother cell, and through a process involving membrane fission the forespore becomes bound by a double membrane. During late sporulation, a peptidoglycan cortex is formed, and the final stage involves the release of the mature spore by the mother cell. When nutrients become available, the spores can germinate and resume the vegetative cycle. The sporulation protein SpoIIE plays an important role in generating the septum (asterisk). Figure modified from Tan and Ramamurthi (2014).

Domesticated strains of *B. subtilis* 168 readily undergo sporulation on NGM (Gusarov *et al.*, 2013; Laaberki and Dworkin, 2008; Smolentseva *et al.*, 2017), indicating shortage of the necessary nutrients for vegetative growth. The precise influence of spores on the physiology of *C. elegans* has not been well defined. Spore formation possibly makes a small contribution to the beneficial effect of *B. subtilis* versus *E. coli* on lifespan in *C. elegans* (Sánchez-Blanco *et al.*, 2016). In one study, nematodes grown on wild-type spores of *B. subtilis* were smaller, thinner and appear to be less fecund than nematodes fed on vegetative cells (Laaberki and Dworkin, 2008). This presumably arises because spores are able to provide less nutrition than vegetative cells, as they can survive the action of the pharynx muscles and pass through the nematode. Despite this, in a separate report, the correlation

between spore formation in a number of bacterial species and the edibility of these species for *C. elegans* (with less edible strains prolonging larval development rate) was relatively poor (Avery and Shtonda, 2003). More recently, *C. elegans* were sustained entirely on *B. subtilis* spores (Donato *et al.*, 2017), indicating that they represent an adequate food source. The spores were able to germinate within the nematode intestine, rather than simply being passed through the nematode, and increased the lifespan of *C. elegans.* The quality of spores as a food source for *C. elegans* is likely to vary depending on genetic differences between strains in pathways affecting the efficiency of spore germination or outgrowth (Donato *et al.*, 2017).

Fig. 1. 5 summarises the host-microbiota interactions discussed in this chapter.

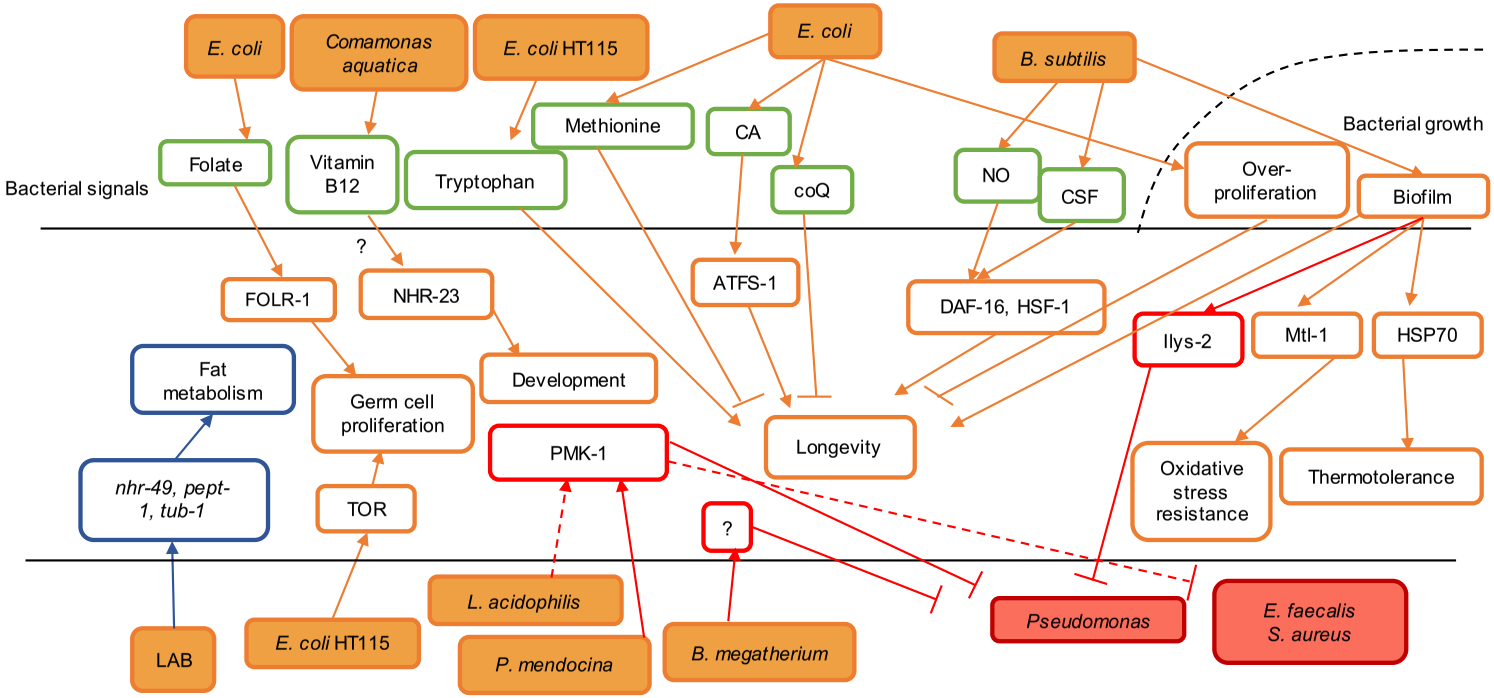



**Figure 1. 5 Host-microbiota interactions in *C. elegans***. The figure summarises the *C. elegans* host-microbiota discussed in this work. The effect of lactic acid bacteria (LAB), *E. coli* and *Bacillus* species on signalling pathways in *C. elegans*, including target of rapamycin (TOR) and insulin signalling, is depicted. Bacterially-derived signals (in green) and features of bacterial growth (over-proliferation or biofilm formation) influence various processes including fat metabolism, germ cell proliferation, development, thermotolerance, heat stress resistance and longevity in *C. elegans*. The expression of folate receptors (FOLR1) and nuclear hormone receptors (e. g. NHR-23) mediate some of these effects. The effects of colonic acid (CA) released by *E. coli* on longevity are mediated by ATFS-1, the host unfolded protein response ($UPR^{mt}$) transcription factor. Pathways in red relate to immunity and resistance to pathogenic bacteria (red fill). Various LAB alter the expression of obesity-related genes (in blue). The effect of microbial interactions on longevity, germ cell proliferation, development and stress resistance are shown in orange. See main text for details. Figure adapted from Gerbaba *et al.* (2017).

## 1.6 Glucose, lifespan and health in *C. elegans*

### 1.6.1 *C. elegans* as a model for understanding glucose toxicity

In humans, diets with a high glycemic index (GI), which are easily metabolized to glucose, have been associated with diabetes, obesity, cardiovascular diseases and age-related diseases (Aston, 2006; Chiu *et al.*, 2011). Similarly, health is compromised by excess glucose intake in model organisms including yeast and *C. elegans.* Although *C. elegans* is capable of using glucose in the medium effectively as an energy source, a number of studies have shown that glucose can exert toxic effects when nematodes are fed on *E. coli* (Lee *et al.*, 2009; Schulz *et al.*, 2007), (Choi, 2011; Mondoux *et al.*, 2011; Schlotterer *et al.*, 2009). Moreover, levels of glucose which are relevant for understanding hyperglycemia-associated conditions in humans similarly exert toxicity in *C. elegans* (Schlotterer *et al.*, 2009).

Glucose reduces lifespan in a range of concentrations (Lee *et al.*, 2009; Schlotterer *et al.*, 2009; Schulz *et al.*, 2007), and this occurs in a dose-dependent manner (Choi, 2011). However, the effect of similar glucose concentrations on *C. elegans* physiology is often variable across studies. A glucose concentration of 0.1% (5.6 mM) is variously reported to reduce (Lee *et al.*, 2009; Schulz *et al.*, 2007), have no effect (Tauffenberger *et al.*, 2012), or even increase the lifespan of *C. elegans* at 25°C (Brokate-Llanos *et al.*, 2014). In contrast, one study reported that 0.01% glucose was sufficient to reduce lifespan (Choi, 2011). In another study (Mondoux *et al.*, 2011), a threshold for glucose stress was defined by assaying the effect of different glucose concentrations on brood size. Here, a concentration of 250 mM (4.5%) was determined to be the threshold beyond which glucose exerted negative effects on fertility. The conditions under which glucose is detrimental appear to depend heavily on factors including temperature (which influences the infectiousness of *E. coli,* amongst other things), the state of the bacterial food (e.g. whether they have been antibiotic arrested) and the presence of 5-fluorodeoxyuridin (FUdR), a chemical that prevents egg hatching.

Several studies using *C. elegans* have demonstrated that the negative effect of glucose on lifespan and health is direct, i.e. it is not mediated by the bacteria (*E. coli* OP50). This is most convincingly shown by the observation that glucose reduces lifespan to a comparable extent whether *C. elegans* is fed on lawns that

have been UV- or heat- killed to prevent metabolic activity or on living lawns (Seo *et al.*, 2018), and that glucose is life-limiting even when the bacteria are incapable of glucose uptake (Lee *et al.*, 2009). As well as reduced lifespan, glucose feeding negatively affects other aspects of nematode physiology, including motility (Choi, 2011; Liggett *et al.*, 2015), neuronal health (Salim and Rajini, 2017), and the ability to withstand oxygen deprivation (Garcia *et al.*, 2015). *C. elegans* which were exposed to different glucose concentrations from hatching until the L4 stage had a marked reduction in locomotion on agar, as well as reduced brood size, compared to non-exposed controls (Salim and Rajini, 2014). The body size of glucose-exposed nematodes was, however, increased.

### 1.6.2 Mechanisms underlying the toxicity of glucose

Our understanding of precisely how glucose negatively impacts *C. elegans* lifespan and other health parameters is becoming more complete. A summary of the various mechanisms is given in Fig. 1. 6. Insulin/insulin-like growth factor-1 signalling (IIS), oxidative stress pathways, fatty acid metabolism (Svensk *et al.*, 2016) and apoptosis (Choi, 2011) have all been implicated in the response to glucose. Signalling through IIS seems to be particularly central to the various effect of glucose on nematode health. IIS signalling regulates lipid and carbohydrate metabolism in *C. elegans,* and activation of this pathway has been proposed to underlie the toxicity of glucose (Lee *et al.*, 2009). Glucose was unable to further reduce the lifespan of *daf-16* individuals, or mutants lacking the heat shock transcription factor HSF-1. Interestingly, regulation of the glycerol channel aquaporin-1 by the IIS pathway entirely accounted for reduced lifespan upon glucose feeding (Lee *et al.*, 2009). Glucose toxicity was therefore suggested to arise from shifts in glycerol metabolism that were induced by down-regulation of the *aqp-1* gene.

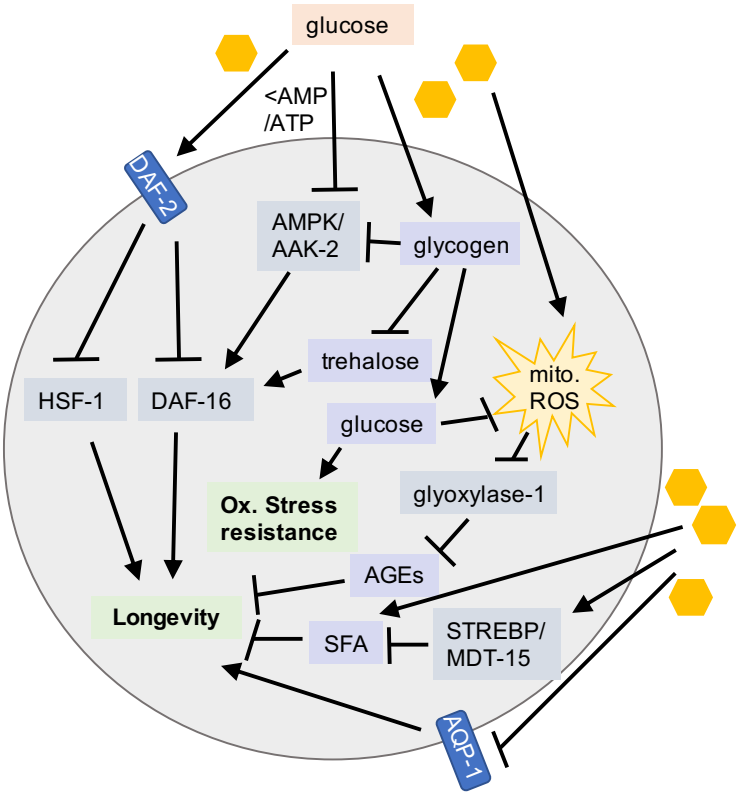



**Figure 1. 6 Summary of mechanisms underlying influence of glucose on lifespan in *C. elegans*.** The pathways mediating the effects of dietary glucose on lifespan and stress response in a *C. elegans* cell are depicted. See main text for details.

A recent study (Gusarov *et al.*, 2017) has suggested that glycogen signalling and IIS are the two primary pathways through which glucose acts to compromise health in *C. elegans.* In *C. elegans,* as in most animals including humans, excess glucose is primarily stored as the branched chain polymer glycogen through the action of the enzyme glycogen synthase, *gsy-1*. Interestingly, *gsy-1* animals show a less dramatic reduction in lifespan in response to high glucose conditions (Seo *et al.*, 2018). Inhibition of the $\alpha$-catalytic subunit of AMPK, AAK-2, by glycogen accumulated on a high glucose diet was identified as a possible mechanism behind reduced nematode lifespan. This glycogen-mediated pathway operates in parallel to but independently of the IIS pathway through *daf-16* to reduce lifespan, as RNAi against *gsy-1* was able to increase the lifespan of *daf-16* mutants fed a high-glucose diet (Gusarov *et al.*, 2017). Interestingly, this AMPK-dependent pathway also mediates enhanced resistance of *C. elegans* to acute and chronic oxidative stress induced by paraquat and diamide in the presence of glucose. Therefore,

perhaps surprisingly, a high glucose diet both reduced lifespan and increased stress resistance in *C. elegans.* Glycogen was identified as the antioxidant mediating this effect and was able to protect *C. elegans* and human hepatocytes from ROS-induced damage (Gusarov *et al.*, 2017).

*C. elegans* can also store glucose in the form of the disaccharide trehalose, which, unlike glucose, is able to extend nematode lifespan when added exogenously (Honda *et al.*, 2010). A recent study (Seo *et al.*, 2018) suggests that the beneficial effects of interfering with glycogen synthase arise from a consequent increase in internal trehalose. The authors propose that a metabolic shift induced by limiting the storage of glycogen leads to an increase in the storage of trehalose, which then increases lifespan and healthspan. These beneficial effects were found to require particular isoforms of DAF-16 and were dependent on DAF-16-dependent upregulation of autophagy. Possibly, an increase in trehalose storage and autophagy also accounts for the findings of Gusarov *et al.* (2017) regarding the positive effects of decreased glycogen synthase activity.

As mentioned, mitochondrial ROS are thought to play a key role in the responses to increased glycolytic flux. The activity of the enzyme glyoxylase-1 is reduced by ROS formation, resulting in an accumulation of the glycolysis-derived glycating agent methylglyoxal (MG). Modification of mitochondrial proteins by MG generates advanced glycation end-products (AGEs) (Schlotterer *et al.*, 2009), leading to the formation of more ROS and possibly reducing the lifespan of *C. elegans.* In one study, over-expressing glyoxylase-1 mitigated the negative effect of glucose on lifespan by limiting the formation of ROS and AGEs (Schlotterer *et al.*, 2009). However, a different study proposed that the longevity of *C. elegans* with a chemically-induced reduction in glucose availability (application of 2-deoxy-D-glucose, 2-DG) arises from an initial increase in mitochondrial respiration and ROS formation. This then induces a lasting hormetic activation of antioxidant defence, and reduced stress levels (Schulz *et al.*, 2007). Similarly, exposure of *C. elegans* larvae to glucose extended lifespan in another report (Tauffenberger *et al.*, 2016), seemingly by activating the $UPR^{mt}$ and leading to hormetic longevity. However, prolonged glucose-induced activation of the $UPR^{mt}$ during adulthood by reduced lifespan. This demonstrates that some level of ROS formation can be beneficial in terms of stress resistance and lifespan, but also highlights the contradictions in the proposed role of ROS formation and oxidant-induced hormesis in the effects of glucose; elevated ROS is cited as a mechanism for increased lifespan upon glucose

restriction (Schulz *et al.*, 2007) as well as reduced lifespan upon glucose exposure (Schlotterer *et al.*, 2009). Alongside its possible role in promoting longevity upon glycogen synthase inhibition, AMPK has been proposed to play a key role in the toxicity of glucose. Inhibition of glycolysis by exposure to a chemical inhibitor lead to an increase in lifespan which was dependent on AAK-2 activity (Schulz *et al.*, 2007).

Various other mechanisms of glucose toxicity in *C. elegans* are being uncovered, including ectopic induction of apoptosis (Choi, 2011) and compromised membrane fluidity as a result of saturated fatty acid (SFA) accumulation (Svensk *et al.*, 2016). Consistent with this, fatty acid desaturation is important for ameliorating chronic glucose exposure (Lee *et al.*, 2009). A key role for the sterol regulatory element-binding protein (SREBP) and mediator-15 (MDT-15) has also been identified. These proteins form a transcription factor complex which promotes the conversion of SFAs accumulated on a high glucose diet into unsaturated fatty acids, the upregulation of which completely alleviated glucose toxicity (D. Lee *et al.*, 2015).

As described, glucose acts directly on *C. elegans* to reduce lifespan. However, the metabolism of the bacterial diet can modulate the effects of glucose on the nematode. In one study, a low concentration – 0.1% – of glucose actually increased *C. elegans* lifespan fed on *E. coli* OP50 at 25 °C (Brokate-Llanos *et al.*, 2014). It was concluded that glucose elicited carbon catabolic repression in *E. coli,* limiting its ability to colonise *C. elegans* and therefore reducing *E. coli*-induced pathogenicity. The higher temperature of 25 °C seemingly exacerbated the level of *E. coli* infection, resulting in a concentration of sugar that is usually detrimental (Lee *et al.*, 2009; Schulz *et al.*, 2007) becoming beneficial. Similarly, the biguanide drug metformin, which is used to treat diabetic patients, was unable to protect *C. elegans* from high levels of glucose as a result of a glucose-induced shift in *E. coli* metabolism (Cabreiro *et al.*, 2013). Thus, the bacterial diet of *C. elegans* is able to modulate the effects of glucose on the nematode.

### 1.7 Bacterial diets lacking ATP synthase and their effect on lifespan in *C. elegans*

As described, a diet of *E. coli* deficient in ATP synthase extends the lifespan of *C. elegans.* $F_1F_0$-ATP synthases (or simply ATP synthases) are multidomain enzyme complexes which couple the electrochemical gradient of protons across

biological membranes with the synthesis of the energy carrier molecule adenosine-5′-triphosphate (ATP). Electrons derived from the reducing equivalents NADH and $FADH_2$ cascade along protein/cytochrome complexes – known as the electron transport chain – before finally reducing molecular oxygen. In the process, protons are pumped across the inner mitochondrial membrane, generating a proton gradient which powers the rotary motion of ATP synthase and generates ATP from ADP and $P_i$. This process, along with the action of ATP synthase, is known as oxidative phosphorylation (Cole, 2016). The *B. subtilis* ATP synthase, located in the cytoplasmic membrane, is encoded by the *atp* operon Fig. 1. 7. This operon is composed of nine genes; eight structural genes which encode subunits of the ATP synthase enzyme complex, and one upstream gene, *atpI,* whose protein product functions to assemble and stabilize the synthase (Preiss *et al.*, 2015). The $F_0$ portion, encoded by *a, b* and *c,* forms a transmembrane proton channel, whilst genes *α, β, γ, δ,* and *ε* comprise the catalytic $F_1$ component.

Bacterial mutants lacking this key enzyme display broad changes in their metabolic profiles. ATP synthase-deficient mutants are not capable of harnessing the transmembrane proton motive force to generate ATP and fail to grow on succinate media as a consequence (Jensen and Michelsen, 1992; Santana *et al.*, 1994). However, mutation of this gene in *E. coli* and *B. subtilis* is non-lethal; mutant strains compensate by increasing metabolic flux through glycolysis and the tricarboxylic acid cycle, thereby generating ATP by substrate-level phosphorylation (Jensen and Michelsen, 1992; Noda *et al.*, 2006). This results in an increased respiration rate, which neutralizes the high production of reducing equivalents and maintains REDOX homeostasis. Growth rate in liquid is compromised in strains of both species lacking the *atp* operon (either the entire operon, or certain subunits) (Santana *et al.*, 1994). As well as having a reduced rate of growth (60-70% of the wild-type strain), mutants of *B. subtilis* lacking parts of the *atp* operon consume twice as much glucose for the same amount of growth as wild-type and produce twice as much acetate as a consequence of increased substrate-level phosphorylation (Santana *et al.*, 1994). Compromised function of ATP synthase leads to increased production of ROS by the mitochondrial respiratory chain (Houštěk *et al.*, 2006; Mracek *et al.*, 2006; Niedzwiecka *et al.*, 2018).

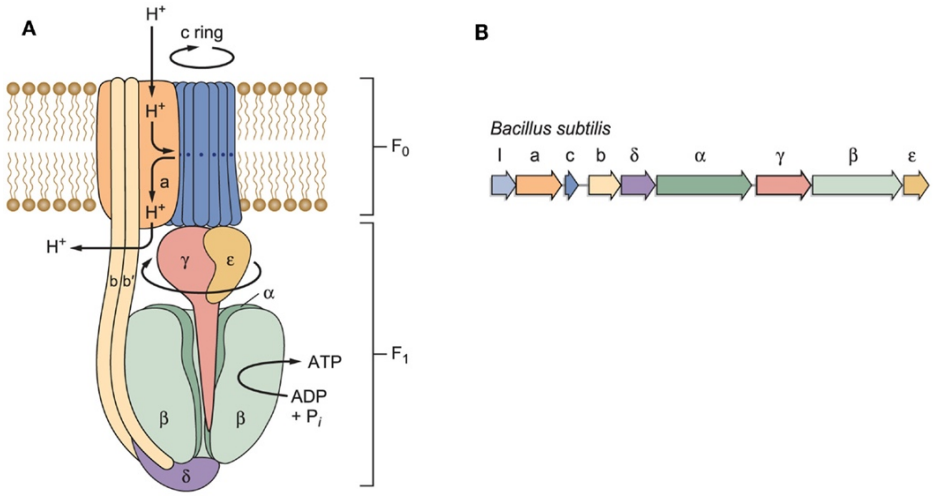



**Figure 1. 7 Structure of the *B. subtilis* $F_1F_0$-ATP synthase (A) and schematic example of the *B. subtilis atp* operon (B).** (A) Each ATP synthase is composed of a membrane-spanning $F_0$ complex and a catalytic soluble component, $F_1$, which functions as a rotor to generate ATP through the action of the α/β catalytic sites. (B) The *B. subtilis atp* operon. Figure adapted from Preiss *et al.* (2015).

As described, the expression of *acdh-1,* which encodes a fatty-acid specific acyl-CoA dehydrogenase (Yuan *et al.*, 2012), in *C. elegans* is downregulated by a diet of life-shortening *Comamonas* DA1877 compared to *E. coli* OP50 (MacNeil *et al.*, 2013). The "dietary sensor" strain *Pacdh-1::gfp* was previously utilised to characterise the nematode pathways regulating differential responses to these diets (Watson *et al.*, 2013). In addition to (as it was eventually discovered) responding to the production of vitamin B12 by *Comamonas* (Watson *et al.*, 2014)*, acdh-1* expression is reduced by dietary restriction (Van Gilst *et al.*, 2005), which can extend lifespan. Since this gene responds to differences in bacterial diet and nutrition that influence lifespan, it was utilised in preliminary studies to identify *B. subtilis* mutants that potentially affect lifespan and ageing in the nematode. A *C. elegans* strain expressing GFP under the promoter for *acdh-1* was screened against a library of *B. subtilis* 168 deletion mutants. Of the mutants in the library, a *B. subtilis* mutant in which the *atp* operon is deleted (*B. s.* Δ*atp*) gave the strongest downregulation of GFP (Jiménez and da Silva, 2015), and this mutant was subsequently shown to increase the lifespan of *C. elegans* by up to 40% (JLB Jiménez, personal communication). These experiments were conducted with glucose added into the NGM to a final concentration of 2%, in order to limit the formation of spores by *B. subtilis.* Interestingly, glucose was required for the mutant to increase lifespan. This stimulated interest in this mutant as a possible probiotic diet which counteracts the toxicity of glucose or turns glucose into a component that

is beneficial to *C. elegans*. The mechanism(s) by which *B. s.* Δ*atp* is able to promote longevity in *C. elegans* is mysterious. Longevity as a result of reduced bacterial growth and gut colonization, a prominent explanation for the beneficial effects of respiratory-defective *E. coli* mutants (Gomez *et al.*, 2012), is unlikely (though it should not be excluded) since *B. subtilis* does not appear to exert pathogenic effects (Garsin *et al.*, 2003; Sánchez-Blanco *et al.*, 2016). This system therefore presents an interesting opportunity to assess the effects of a respiration-deficient diet without the confounding factor of bacterial pathogenicity.

### 1.8 Aims and thesis plan

Broadly, the aim of this work was to uncover the mechanisms by which *B. s.* Δ*atp* in the presence of glucose extends the lifespan of *C. elegans*. This work also extends investigation beyond lifespan to incorporate aspects of health and resistance to stress over time.

The objectives of this thesis were as follows:

1. Understand the role played by glucose in the benefits elicited by *B. s.* Δ*atp*(+Glu).
2. Define whether dietary restriction is responsible for the longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu), and if so how this is elicited.
3. Investigate how the health and stress responses of *C. elegans* are affected by *B. subtilis* lacking ATP synthase, with or without glucose.

In Chapter 3, the effect of respiration-deficient *B. subtilis* 168 on the lifespan, motility and stress response of *C. elegans* in the presence and the absence of glucose is investigated. Specifically, whether a diet of *B. s.* ∆*atp*(+Glu) is required during development, and whether FUdR affects bacterial metabolism and nematode lifespan, is explored further. In Chapter 4, whether *B. s.* ∆*atp*(+Glu) extends lifespan through an active mechanism, such as the release of a pro-longevity metabolite, or through a passive mechanism, such as a change in the physical properties of the bacterial lawn, is addressed. The possibility that ATP synthase-deficient *B. subtilis* induces dietary restriction through reduced food intake receives particular focus as a putative passive mechanism for longevity. Chapter 5 details the development of RNA interference (RNAi) for further probing mutant-responsive effector pathways in

*C. elegans.* Finally, in Chapter 6 a different *B. subtilis* master strain which is incapable of forming spores is constructed. The lifespan, health and stress response of *C. elegans* fed on this strain with ATP synthase either functional or deleted is investigated.

## 2 General Materials and Methods

See separate Materials and Methods sections in each chapter for further chapter-specific details (sections 3.3, 4.3, 5.2 and 6.3).

### 2.1 Materials

#### 2.1.1 Reagents

**M9 buffer**

Dissolve 3 g $KH_2PO_4$, 5 g NaCl, 6 g $Na_2HPO_4$ into $_{dd}H_2O$. Add $_{dd}H_2O$ to 1 litre. Autoclave, and add 1 ml 1M $MgSO_4$.

**Phosphate buffered saline (PBS) 10X (pH 7.4)**

Dissolve 80 g NaCl, 2 g KCl, 14.4 g $Na_2HPO_4$ and 2.4 g $KH_2PO_4$ into $_{dd}H_2O$. Add $_{dd}H_2O$ to 1 litre and adjust pH to 7.4. Autoclave.

**Tris-EDTA (TE) buffer**

Add 1 ml of 1M Tris-Cl, pH 8.0, and 200 µl 0.5M EDTA to 98.8 ml $_{dd}H_2O$.

**Tris-acetate-EDTA (TAE) buffer**

A 50x stock solution (1 L) contained 242 g of Tris base, 57.1 mL glacial acetic acid and 500 mM EDTA (pH 8.0). The Tris was dissolved, and the solution was brought to 1 L in $_{dd}H_2O$. When required for gel electrophoresis, the stock solution was diluted in distilled water to make a 1 x working solution (40mM Tris, 20mM acetic acid, and 1mM EDTA).

**Alkaline hypochlorite solution**

Add 1.5 ml Sodium hypochlorite and 2 ml of 4N NaOH to 7.5 ml $_{dd}H_2O$.

**Sodium azide (1M)**

Dissolve 65 mg of sodium azide powder in 1 ml $_{dd}H_20$. Store at 4 °C until use.

#### 2.1.2 Bacterial growth media

See Table 2. 1. LB or LB-based media were pre-prepared and supplemented with the appropriate antibiotics before use. Paris Medium was

prepared using distilled, deonised water, and was stored away from light at 4 °C. Fresh medium was prepared regularly.

**Table 2. 1 Bacterial growth media**

| Media | Composition |
|---|---|
| LB (Luria-Bertoni) | 0.005 g/ml yeast extract, 0.01 g/ml Bacto-tryptone, 0.01 g/ml NaCl (pH 7.2). |
| LB-agar (LBA) | 0.005 g/ml yeast extract, 0.01 g/ml Bacto-tryptone, 0.01 g/ml NaCl, 0.015 g/ml Bacto-agar (pH 7.2). |
| LBA + 1% starch | 0.005 g/ml yeast extract, 0.01 g/ml Bacto-tryptone, 0.01 g/ml NaCl, 0.015 g/ml Bacto-agar (pH 7.2), 1% (w/v) soluble starch. Autoclave (20 mins, 121ºC). |
| Paris Medium (for *B. subtilis* transformation) | 1x PC, 1% D-glucose, 2 mM K-glutamate.$H_20$, 0.002 mg/ml Fe-ammonium citrate, 0.1 % casaminoacids, 0.04 mg/ml Tryptophane, 3 mM $MgS0_4$ |

LBA was supplemented with antibiotics as shown in Table 2. 2.

**Table 2. 2 Antibiotic supplements of LBA.** Solid LBA was melted in the microwave and the following antibiotics were added once the media was hand-hot.

| Antibiotic | Solvent | Stock concentration | Final concentration |
|---|---|---|---|
| Phleomycin | $H_20$ | 5 mg/ml | 5 µg/ml |
| Erythromycin | 100% EtOH | 2 mg/ml | 2 µg/ml |
| Ampicillin | $H_20$ | 50 mg/ml | 50 µg/ml |

### 2.1.3 Nematode growth media (NGM)

**NGM without glucose (NGM(-Glu)).**

Nematode growth media (NGM) prepared without additional supplements, as given in Table 2. 3, is referred to throughout as NGM(-Glu). A peristaltic pump (INTEGRA Biosciences) was used to dispense 9 ml of liquid NGM into 6 cm petri dishes (VWR). Plates were left at room temperature to dry for three to five days, after which they were stored at 15 °C until use (up to two weeks).

**Table 2. 3 Nematode growth medium without glucose (NGM(-Glu)).**

| Name | Source | Stock MW or concentration | Final concentration | Weight or Volume * |
|---|---|---|---|---|
| NaCl | Fisher Scientific | | | 1.2 g |
| BD Bacto™-peptone | BD Biosciences | | | 1 g |
| Difco™ Agar, granulated | BD Biosciences | | | 8 g |
| Magnesium sulphate | Fisher Scientific | 1 M | 1 mM | 400 ul |
| Cholesterol | Sigma-Aldrich | 5 mg/ml | 5 mg/L | 400 µl |
| Calcium Chloride | Fisher Scientific | 1 M | 1 mM | 400 ul |
| Potassium Phosphate pH6 | Fisher Scientific | 1 M | 25 mM | 10 ml |
| Nystatin | Sigma-Aldrich | 10 mg/ml in 75% EtOH | 10 µg/ml | 400 µl |
| * For a final volume of 400 ml NGM(-Glu). | | | | |

The NaCl, bacto-peptone and agar were added to 390 ml $_{dd}H_20$, and the mixture was autoclaved. The remaining ingredients were added after the solution was allowed to cool to around 55 °C.

**NGM containing glucose (NGM(+Glu))**

For NGM(+Glu), add 8 g of D(+)-Glucose Anhydrous (Fisher Scientific, AR grade) as a powder into liquid NGM(-Glu) and dissolve completely prior to pouring plates, for a final concentration of 2%.

**NGM for growth of *E. coli* OP50-1**

Prepare the NGM(-Glu) as above. However, also add streptomycin at a final concentration of 50 µg/ml into NGM(-Glu) before pouring (400 µl).

### 2.1.4 *C. elegans* strains

The wild-type N2 (Bristol) *C. elegans* strain was used throughout unless otherwise indicated. A list of mutant strains that were used in this thesis is given in Table 2. 4. Mutant strains were provided by the *Caenorhabditis* Genetics Center

(CGC, University of Minnesota), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

**Table 2. 4 List of mutant and transgenic overexpressing *C. elegans* strains used in this study**

| Strain | Genotype [description] | Phenotype(s) | Described in | Source | Chapter(s) |
|---|---|---|---|---|---|
| APS9 | (*vsIs33 [dop-3::rfp] ; wwIs24 [Pacdh-1::gfp + unc-119(+Glu)]*) | DOP-3::RFP expressed in body wall muscles; GFP expression mainly in distal region. | MacNeil *et al.* (2013) | Jiménez and da Silva (2015) | 3 |
| SJ4100 | *zcIs13 [hsp-6::gfp]* | Expression of HSP-6::GFP throughout body | Yoneda *et al.* (2004) | CGC | 3 |
| CL2166 | *dvIs19 [(pAF15)gst-4p*::*gfp::NLS] III* | Expression of GST-4::GFP largely in tail. | Link and Johnson (2002) | CGC | 3 |
| DA1116 | *eat-2(ad1116) II.* | Reduced pharynx pumping; long-lived | Avery (1993) | CGC | 4 |
| TJ356 | *zIs356 [daf-16p::daf-16a/b::gfp + rol-6(su1006)]* | Dauer constitutive (Daf-c); Roller; Fluorescent DAF-16::GFP | Lin *et al.* (2001) | CGC | 6 |

### 2.1.5 *E. coli* strains

The B-type *E. coli* strain OP50-1, a streptomycin-resistant uracil auxotroph (Brenner, 1974) was used as a diet for all nematode handling and maintenance

prior to synchronisation of nematode cultures for experimentation. Occasionally, *E. coli* OP50-1 was also provided to *C. elegans* as an experimental diet post-synchronisation, as a comparison with nematodes fed on *B. subtilis.*

Table 2.5 shows the other *E. coli* strains used in this thesis, in the indicated chapter. These strains (DK8 and DK8 Δ*unc*) were solely provided as experimental diets to *C. elegans* following nematode synchronization from *E. coli* OP50-1 stock plates. Strain DK8 was derived from the *E. coli* K-12 strain 1100 (Humbert *et al.*, 1983)

**Table 2. 5 *Escherichia coli* strains.**

| Strain | Genotype | Originally described | Source | Antibiotic resistance (concn, µg/ml) | Chapter |
|---|---|---|---|---|---|
| DK8 | Strain DK8 Δ*unc* (below) harboring plasmid pBWU13 containing the wild-type ATP synthase (*unc*) operon | Iwamoto *et al.* (1991) | Prof. Z. Ahmad (Kirksville College of Osteopathic Medicine, Kirskville, MO, USA). | Amp(100) | 3 |
| DK8 Δ*unc* | Δ*uncB-C, ilv*::Tn 10 | Klionsky *et al.* (1984) | | Amp(100) | |

### 2.1.6 *B. subtilis* strains

The *B. subtilis* strains used in this thesis were obtained from Dr Emma Denham (Division of Microbiology and Infection, Warwick Medical School, University of Warwick). The *B. subtilis* master strain was ultimately derived from *B. subtilis* strain 168 by deletion of large dispensable regions, namely the prophages SPβ and PBSX and the prophage-like element *skin* (Westers *et al.*, 2003). The resulting strain (TF8A) was further modified by (Tanaka *et al.*, 2013) by replacement of the *upp* counter-selection marker with a neomycin resistance gene (TF8A λPr-*neo::Δupp*). This strain is referred to as *B. s.* MS herein. An ATP synthase deletion was introduced into TF8A λPr-*neo::Δupp* by targeted homologous recombination with a DNA cassette (*upp-phleo-cI*) (Tanaka *et al.*, 2013). Whole genome

sequencing of the mutant (E. L. Denham) confirmed that the entire ATP synthase operon (Δ*atpA-I* ) was deleted in this strain. Herein, this strain is referred to as *B. s.* Δ*atp.* Strains *B. s.* MS and Δ*atp* were used throughout this work unless otherwise stated.

The gene *rncS* was deleted in both of these strains to generate strains *B. s.* MS Δ*rncS* and *B. s.* Δ*atp,* Δ*rncS.* These strains were used in Chapter 5 to construct RNAi-competent strains.

As described in Chapter 6, a different strain of *B. subtilis,* 168 *trpc2,* was transformed in order to generate the spore-less strain 168 MS. Δ*atpA-I* was also deleted in this strain to generate strain *B. s.* 168 Δ*atp*. Further information about strain construction is given in the indicated chapter. Strain details are given in Table 2.6.

**Table 2. 6 *Bacillus subtilis* strains used in this work.** Further information about each strain is given in the indicated chapter. BGSC, Bacillus Genetics Stock Center.

| Strain | Genotype | Source | Characteristics | Antibiotic (concn, µg/ml) | Chapter(s) |
|---|---|---|---|---|---|
| *B. s.* Master Strain (*B. s.* MS) | *trpc2*; ΔSPβ; Δ*skin*; ΔPBSX; Pr-*neo*:: Δ*upp* | Tanaka *et al.* (2013). | Lacks regions of the chromosome relative to strain 168. | - | 3,4 |
| *B. s.* Δ*atp* | *trpc2*; ΔSPβ; Δ*skin*; ΔPBSX; Pr-*neo*:: Δ*upp*; *ΔatpA-I* | Tanaka *et al.* (2013). | *B. s.* MS lacking ATP synthase (*atp*) operon. | Phleo (5) | 3,4 |
| *B. s.* MS Δ*rncS* | *trpc2*; ΔSPβ; Δ*skin*; ΔPBSX; Pr-*neo*:: Δ*upp*; Δ*rncS* | E. L. Denham (Warwick Medical School, University of Warwick). | *B. s.* MS lacking RNase III. | - | 5 |
| *B. s.* Δ*atp*, Δ*rncS* | *trpc2*; ΔSPβ; Δ*skin*; ΔPBSX; Pr-*neo*:: Δ*upp*; Δ*rncS*; Δ*atpA-I* | | *B. s.* MS lacking RNase III and ATP synthase (*atp*) operon. | Phleo (5) | 5 |
| *B. s.* 168 *trpc2* | *trpc2, derived from 168* | Laboratory stock (BGSC) | Tryptophan-deficient laboratory strain. | - | 6 |
| *B. s.* 168 Master Strain (168 MS) | *trpc2*; Δ*spoIIE* | This study | Strain 168 lacking SpoIIE; incapable of spore formation. | Ery (2) | 6 |
| *B. s.* 168 Δ*atp* | *trpc2*; Δ*spoIIE*; Δ*atpA-I* | This study | *B. s.* 168 MS (incapable of spore formation) lacking ATP synthase (*atp*) operon. | Ery (2), Phleo (5) | 6 |

## 2.2. Methods

### 2.2.1 Bacterial growth and storage

#### 2.2.1.1 Preparation of glycerol stocks

Glycerol stocks were prepared using equal volumes of 80 % glycerol and overnight bacterial culture. Stocks were mixed thoroughly and stored at -80°C for long-term storage of bacteria.

#### 2.2.1.2 Temporary storage of bacteria

Glycerol stocks were used to streak bacteria onto LB agar containing the appropriate antibiotic. Plates were incubated at 37 °C overnight. LB plates streaked with *B. subtilis* strains were stored at 15 °C for up to three weeks. Plates containing *E. coli* OP50-1 were stored at 4 °C for up to one month.

#### 2.2.1.3 Overnight cultures

Single colonies were picked to aseptically inoculate LB containing the appropriate antibiotic. *E. coli* OP50-1 cultures were left to grow overnight at 20 °C, and were subsequently stored at 4 °C until use. Cultures of all other bacterial species/strains were inoculated from LB plates and grown at 37 °C overnight, with shaking at 200 rpm. These cultures were not stored, but were used within 24 hours for seeding NGM plates.

#### 2.2.1.4 Seeding of NGM(-Glu) with *E. coli* OP50-1 (for nematode maintenance)

A multi-pipettor was used to seed 50-100 μl droplets of *E. coli* OP50-1 onto NGM(-Glu) plates. These plates were left to dry at RT overnight, and were subsequently stored at 15 °C until use (up to one month).

### 2.2.2 Polymerase Chain Reaction (PCR)

Nucleic acids were amplified by PCR. The standard PCR mixture (20 μl) in this work was prepared using GoTaq Green Master mix, 2 X (Promega) in microcentrifuge tubes according to the following recipe:

10 μl GoTaq Green Master mix, 2 X
1 μl template DNA (< 250 ng)

5 μM forward and reverse primer

5 μl nuclease-free $H_20$

An Eppendorf™ Mastercycler™ pro PCR System was used for all PCR reactions. Specific cycling conditions varied according to the primer set. General cycling parameters are shown in Table 2. 7.

**Table 2. 7 General PCR thermocycling conditions.** Steps 2-4 repeated for x34 cycles.

| **1. Initial denaturation** | **2. Denaturation** | **3. Annealing** | **4. Extension** | **5. Final extension** |
|---|---|---|---|---|
| 95°C<br>1 min | 95°C<br>30 seconds | 55°C<br>30 seconds | 72°C<br>45 seconds | 72°C<br>5 min |

### 2.2.3 Quantitative real-time PCR (qRT-PCR)

Gene expression levels were determined using an Agilent Mx3000P Real Time PCR machine (Thermo Fisher Scientific) and associated qRT-PCR kit. Each separate reaction contained SYBR Green Master Mix, 1 μl of each primer (5 μM) and nuclease-free $H_20$ to a final reaction volume of 20 μl. Reactions were performed in technical triplicate in 96-well plates using the thermocycling setup shown in Table 2. 8. Quantification was performed using the comparative Ct method. The difference between the mean Ct value of a sample was subtracted from that of the indicated housekeeping control ($\Delta Ct = Ct_{sample} - Ct_{housekeeping}$). Relative expression is given as 2^-ΔCt (Chapter 5), or as 2^-ΔΔCt (Chapter 4), where $\Delta\Delta Ct = \Delta Ct_{sample}$ - mean ΔCt of *C. elegans* fed on *B. s.* MS(-Glu).

**Table 2. 8 qRT-PCR thermocycling conditions.** Steps 2-4 repeated for x40 cycles.

| **1. Initial denaturation** | **2. Denaturation** | **3. Annealing** | **4. Extension** | **5. Final extension** |
|---|---|---|---|---|
| 95°C<br>10 min | 95°C<br>30 seconds | 60°C<br>30 seconds | 72°C<br>30 seconds | 72°C<br>5 min |

SYBR Green fluorescence was normalised using the ROX fluorescent dye as an internal reference. Each run also included no template controls (NTCs) and no reverse transcriptase controls (NRTs) to ensure no contamination by exogenous sources or genomic DNA, respectively.

### 2.2.4 Gel electrophoresis of nucleic acids

DNA molecules were separated by size using agarose gel electrophoresis. 0.8-1% agarose gels were prepared in 1X TAE buffer containing GelRed™ Nucleic Acid Stain. Gels were cast in a gel tank and allowed to set after adding a gel comb. 6X Purple Gel Loading Dye (NEB) was used to load samples into the gel, unless the samples to be loaded were derived from a PCR in which Go Taq® Green Master Mix (Promega) was used. Electrophoresis was performed in 1X TAE at 80V for 45-80 minutes depending on the required band separation. Gels were visualised using ultraviolet light in a gel doc system.

### 2.2.5 Gibson Assembly

Gibson assembly ® (NEB) was utilised in order to join DNA fragments in a single isothermal reaction. All Gibson reactions were carried out at 50 ºC for 45 minutes. More details are given in the relevant chapters. 5-alpha Competent *E. coli* was transformed with the assembled product (2 μl) according to manufacturer's instructions (NEB). The transformed cells were selected onto LB + Amp plates overnight at 37 ºC. The following day, colonies were observed, and plasmids were isolated.

### 2.2.6 Single colony PCR

Following Gibson assembly reactions, colony PCR was carried out on positive transformants as follows. Reactions were set up in PCR tubes, and a pipette tip was used to inoculate each tube with a different positive colony growing on ampicillin selection plates.

PCR reactions were set up containing 1 μl each of the relevant primers, 10 μl of GoTaq Green Master Mix (Promega) and $_{dd}H_20$ to a final volume of 20 μl. Colonies were then gently touched with a pipette tip and introduced into each PCR reaction. Control reactions that were not inoculated with a colony were also set up. Instead, the LBA was gently touched with a tip and pipetted into the mix. This

indicated the level of background fragment from the preceding Gibson reaction that had been introduced onto the plate. Colonies which were confirmed to contain the relevant plasmid construct were inoculated into LB medium plus ampicillin (100 µg/ml), and grown overnight at 37 °C, 200rpm. 2 ml of each culture was then used for the purification of plasmid DNA using the QIAprep Spin Miniprep Kit (QIAGEN).

### 2.2.7 Transformation of *B. subtilis*

Single colonies of the *B. subtilis* strain to be transformed were picked from LB plates and inoculated into 500 µl of Paris Medium (PM). These were grown overnight at 37 ºC, with shaking 200 rpm. The following day, 10 µl of the overnight cultures were pipetted into 500 µl PM and incubated at 37ºC for 3 hours, 200 rpm. 10 µl of the relevant DNA was then added into the culture, and alongside this a negative control without any DNA was included. These cultures were incubated at 37 ºC for 5 hours, at which point 166 µl were spread using sterile beads onto LB-agar plates with the appropriate antibiotic (Table 2. 6). These were grown at 37ºC overnight, at which point positive colonies were observed.

### 2.2.8 Synthesis of complementary DNA (cDNA)

Prior to qRT-PCR analysis, cDNA was generated from total RNA extracted from bacterial or nematode samples. Genomic DNA was first removed from these samples by treatment with DNase I. For 50 ul of RNA, 5ul of 10 x DNase reaction buffer and 5 ul of DNase1 AMPD1 (Sigma) were added into each sample, and these were incubated for 15 min at RT. 5 ul of stop solution (Sigma) was then added, and samples were incubated at 70 °C for 10 min. A Nanodrop machine (Implen Nanophotometer NP80) was then to quantify the RNA concentration. 0.5-2.0 ug of RNA was added into a 1.5 ml Eppendorf tube and made up to 14 ul with nuclease-free water. Approximately the same amount of RNA was used to synthesise cDNA, for each sample. 1 ul of random primer was added into each sample and mixed gently. Samples were heated at 70 °C for 5 min to melt secondary structures, and immediately cooled on ice to prevent secondary structures reforming.
After a 5 min incubation on ice, first strand cDNA was synthesised using RETROscript Kit (Invitrogen). Samples were incubated for 1 hr at 37 °C with 5 µl of MMLV buffer, 1.25 µl of dNTPs, 1 µl of MMLV reverse transcriptase (RT) enzyme, 0.5 µl of RNasin (RNase inhibitor) and 2.25 µl of RNase free water (25 µl reactions), and were stored at -20 °C until required for qRT-PCR.

### 2.2.9 Nematode handling and maintenance

#### 2.2.9.1 Maintenance of *C. elegans* stocks

Unless otherwise stated, *C. elegans* were maintained on a lawn of *E. coli* on 6 cm NGM plates kept at 20 °C. Nematodes were transferred onto fresh plates periodically to prevent starvation. All experiments were conducted at 20 °C unless otherwise stated.

#### 2.2.9.2 Nematode synchronisation by sodium hypochlorite treatment

Synchronization with a bleaching protocol is used to obtain large numbers of nematodes at the L1 stage. The procedure was carried out as described in Porta-de-la-Riva *et al.* (2012) (Protocol B), with centrifugation steps at 1500 rpm for 5 mins. M9 buffer was used during washing steps.

#### 2.2.9.3 Introduction of nematodes onto the bacterial diets

Nematodes were synchronized and introduced as L1s (resuspended in M9) onto NGM plates seeded with the relevant diet as described. With the indicated exceptions, these were NGM(-Glu) plates, and post-embryonic development therefore occurred in the absence of glucose. 150-200 L1s were introduced onto each seeded plate. The beginning of the experiment (day 0) was designated as the mid-late L4 larval stage (48-54 hrs later). Upon reaching this stage, nematodes were transferred onto either NGM(-Glu) or NGM(+Glu) plates seeded with the same diet. All animals therefore experienced only one diet throughout their lives. The first day of egg laying represents day 1 of adulthood in all experiments. The same procedure was used for *B. subtilis* and *E. coli* diets.

L1s that had been introduced onto *B. s* Δ*atp* were delayed in development and took longer to reach the mid-L4 stage. Therefore, synchronized larvae were monitored until the majority of individuals reached the mid-L4 stage, to ensure that all individuals were exposed to glucose at approximately the same stage of development. This usually took 3-6 hours after *B. subtilis* MS-fed individuals were transferred onto experimental plates. Subsequent recordings of nematode physiology throughout their life (e.g. thrashing, fluorescent reporter expression) were made with respect to this delay, so that nematodes fed on *B. s.* Δ*atp* were measured later in the day than those fed on MS. No developmental delay was

observed in *C. elegans* fed on spore-less *B. s.* 168 Δ*atp* (Chapter 6). Therefore, these individuals were transferred as L4s onto NGM(-Glu) and NGM(+Glu) at the same time as animals fed on *B. s.* 168 MS.

#### 2.2.9.4 Immobilization of nematodes for imaging

At the indicated timepoints, *C. elegans* were immobilized in 10 μl 0.5 M sodium azide solution pipetted directly onto the surface of an NGM plate. Nematodes were manually aligned using a platinum wire pick.

### 2.2.10 Microscopy

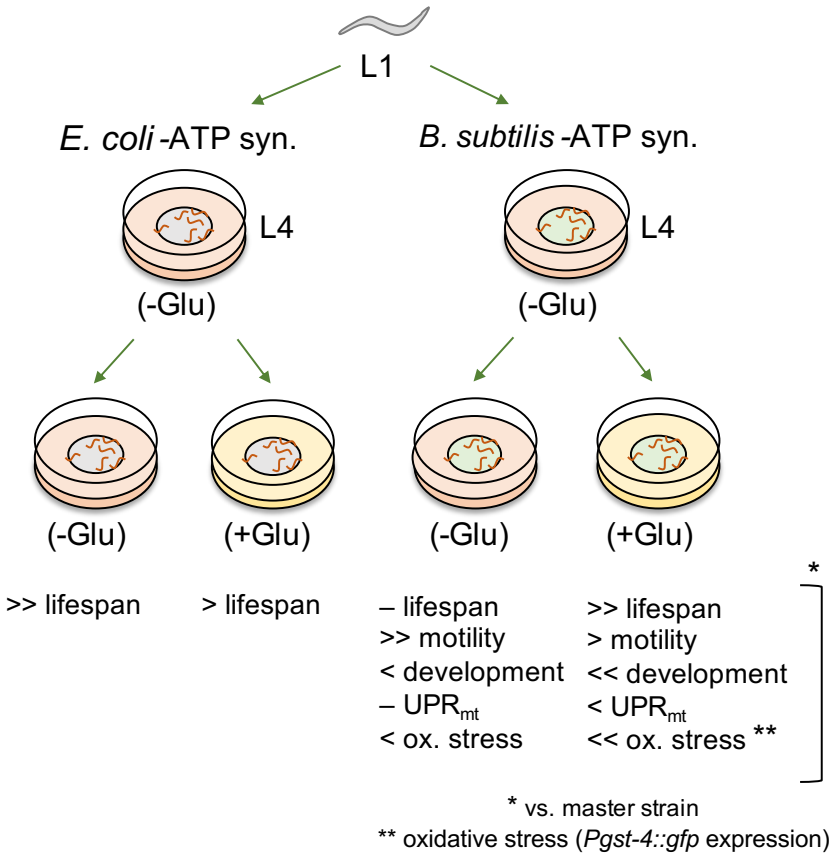
General *C. elegans* maintenance as well as life span, fecundity and body size observations were performed using an Olympus SZ61 stereo microscope**.** Occasionally, an Olympus SZX7 was used for imaging. For higher resolution and fluorescence imaging, a Zeiss Axio Zoom V16 Fluorescence microscope was used (Carl Zeiss Microscopy, Thornwood, NY, USA).

### 2.2.11 Statistical analysis (general)

All data were first inputted into Microsoft Excel in the appropriate format and saved as comma separate value files. These data were imported into R ver. 3.1.1 for further handling. Statistical testing and data visualization was used to determine whether the data belong to a normal distribution (Shapiro-Wilk test, Q-Q plots), and whether the data were derived from populations with the same shape of distribution (Levene's Test, residual plots). When the assumptions of normality and homoscedasticity were met, One-Way ANOVA was used with Tukey's HSD *post-hoc* test. If the data were normal but did not have equal variance, Welch ANOVA followed by the Games-Howell *post-hoc* test was employed. For non-normal data with unequal variance, the Kruskal-Wallis test with Dunn's *post-hoc* test (Benjaminyi-Hochberg FDR multiple comparison) was used to compare mean ranks.

## 3 Characterisation of lifespan, health and stress responses in *C. elegans* fed on ATP synthase-deficient *Bacillus subtilis.*

### 3.1. Graphical abstract




### 3.2 Introduction and aims

Here, the effect of a mutant of *B. subtilis* lacking ATP synthase on *C. elegans* lifespan, health, and stress reporter expression was investigated in the presence and absence of dietary glucose. The aims of the experiments in this chapter were to:

- Investigate the influence of changing parameters including timing of glucose exposure, the preparation of the bacteria (concentrated versus unconcentrated) and the addition of 5-fluorodeoxyuridine (FUdR) on longevity induced by *B. s.* Δ*atp*(+Glu)
- Define the effect of *B. s.* Δ*atp*(-Glu) on the lifespan of *C. elegans*
- Begin to understand how *C. elegans* health and stress response is affected by the diets

## 3.3 Materials and Methods

### 3.3.1 Reagents

#### 3.3.1.1.1 5-fluorodeoxyuridin (FUdR) solution

Weigh 24.6 mg of FUdR and add to 1 ml of $ddH_20$ to make a 100 mM stock solution. Store solution at – 20 °C.

#### 3.3.1.1.2 Paraquat solution

Prepare 1 M paraquat solution by dissolving 0.257 g of paraquat powder in 1 ml $_{dd}H_20$.

### 3.3.2 Nematode growth medium (NGM)

**NGM containing 5-fluorodeoxyuridine (FUdR)**

Add 400 µl of a 100 mM stock of FUdR into liquid NGM(-Glu) or NGM(+Glu) for a final concentration of 100 µM FUdR.

**NGM for growth of *E. coli* DK8 and DK8 Δ*unc***

Prepare NGM(-Glu) and NGM(+Glu) as described in Chapter 2.

### 3.3.3 *C. elegans* strains

The wild-type N2 (Bristol) strain was used unless otherwise indicated. The mutant strains APS9, SJ4100 and CL2166 were also utilised (see Table 2. 4, Chapter 2).

### 3.3.4 *B. subtilis* strains

*B. s.* MS and *B. s.* Δ*atp* were used throughout (see Table 2. 6, Chapter 2).

### 3.3.5 Growth of bacteria on NGM

#### 3.3.5.1 Concentrating *B. s.* MS and Δ*atp* lawns

50 ml LB overnight cultures of *B. s.* MS and Δ*atp* were prepared in autoclaved 250 ml glass flasks. Following overnight incubation, the optical density

($OD_{600}$) of each culture was measured with a UV spectrophotometer at 600 nm (Thermo Scientific). Measurements were made in triplicate and averaged. Based on these values, the $OD_{600}$ of each culture was equalised by the addition of sterile LB to the *B. s*. MS culture, which invariably grew more than the mutant (by 2-3 x). *B. s.* Δ*atp* was concentrated 60 x as follows. A 50 ml overnight culture was centrifuged at 5000 rpm for 5 mins. The supernatant was carefully discarded, and the pellet was resuspended in 500 µl LB. Measurement of $OD_{600}$ after this procedure confirmed that the bacteria were 60 x concentrated. Although it grew well, in the indicated experiments *B. s*. MS was also concentrated by the same procedure, to control for possible effects of preparing concentrated lawns.

#### 3.3.5.2 Seeding NGM containing FUdR

Nematodes were only exposed to FUdR from L4 stage onwards as this chemical interferes with nematode development. Therefore, NGM(-Glu) and (+Glu) plates containing 100 µM FUdR were seeded with a 50 µl spot of overnight culture. NGM(+Glu) was seeded once, whereas NGM(-Glu) were seeded once, left to dry, and reseeded. FUdR also impaired the growth of the bacterial lawn. Therefore, plates were left to grow for two days prior to the introduction of nematodes.

#### 3.3.5.3 Seeding NGM lacking FUdR

To seed NGM(+Glu), overnight cultures of *B. s.* MS and Δ*atp* were diluted approximately 3x by adding 300 µl of the culture to 700 µl $_{dd}H_20$. 50 µl of this dilution was seeded onto NGM(+Glu) plates. To seed NGM(-Glu) plates, bacteria were either concentrated or unconcentrated, as indicated. 50 µl of concentrated bacteria were seeded onto NGM(-Glu) plates. Alternatively, plates were seeded with 50 µl of *B. s.* MS and 50 µl of *B. s.* Δ*atp* culture directly, without concentrating (referred to as unconcentrated lawns) onto NGM(-Glu). *B. s.* Δ*atp* was seeded once, left to dry at room temperature, and reseeded, and the procedure was repeated (3 x seeded, 150 µl total). Following seeding, all plates were left to dry and grow at 20°C for 24 hrs before the introduction of nematodes.

#### 3.3.5.4 Seeding NGM with *E. coli* OP50-1 (for stress reporter screens)

NGM(-Glu) and NGM(+Glu) was seeded with 50 µl of *E. coli* OP50-1 direct from a stock culture kept at 4ºC. All plates were left to grow at 20 °C overnight.

#### 3.3.5.5 Seeding NGM with *E. coli* DK8 and DK8 Δ*unc*

Overnight cultures of *E. coli* DK8 and DK8 Δ*unc* were concentrated prior to seeding, using the same methodology as described above. Unlike *B. subtilis* experiments, the bacteria were also concentrated prior to seeding onto NGM(+Glu) plates, owing to poor growth of *E. coli* DK8 Δ*unc* even +Glu. 50 μl of concentrated bacteria was seeded on NGM(-Glu) and NGM(+Glu) plates. Plates were dried at RT and stored at 20 °C to grow overnight.

### 3.3.6 Nematode experimentation

Throughout the experiments in this chapter, *C. elegans* were maintained at a density of 20-25 nematodes per NGM plate.

#### 3.3.6.1 Imaging of *Pacdh-1::gfp* expression

*C. elegans* strain APS9 were synchronised and introduced onto the indicated diet. As a control to demonstrate reporter downregulation upon starvation, nematodes were fed on *B. s.* MS(-Glu) until L4, at which point they were transferred onto NGM(-Glu) plates lacking bacto-peptone and with the addition of 0.7 μg/ml chloramphenicol in the medium to limit bacterial growth. Nematodes were immobilised at day 2 of adulthood, and GFP (excitation: 470/40nm; emission: 525/50nm) images were taken of day 2 adults at 32x magnification, with a constant exposure time.

#### 3.3.6.2 Lifespan assays

*C. elegans* was introduced onto each of the diets as described in section 2.2, Chapter 2. Animals were scored as alive, dead or censored (escape from the plate, extrusion of the intestines from the vulva (vulval explosion) or the internal hatching of larvae (bagging)) every day and transferred every other day onto fresh plates until the end of the experiment, to move them away from their progeny and to compensate for any depletion of food or glucose from the plates. If nematodes had stopped moving, they were gently prodded with a platinum wire for scoring.

For experiments in the presence of FUdR, L1s were introduced onto NGM plates without FUdR until the L4 stage, at which point they were transferred onto NGM(-Glu) or NGM(+Glu) with 100 μM FUdR (nematodes fail to develop in the presence of FUdR). Nematode transfer occurred every two days until day 18 of adulthood, and every four days following this.

#### 3.3.6.3 Measurement of GFP in stress reporter lines

SJ4100 and CL2166 animals were synchronised in large quantities. Post-embryonic development (L1-L4) took place without glucose. Animals on glucose plates had therefore only experienced glucose conditions for 15 hrs at the time of imaging. The paraquat positive control was prepared as follows. 50 µl of a 200 mM paraquat solution was pipetted onto two NGM(-Glu) plates seeded with *E. coli* OP50-1 the previous day. This was left to soak into the plate and dry for 6 hrs, after which 20 SJ4100/CL2166 worms were moved onto each plate. Transfers took place in parallel with the other conditions. The paraquat plates and x2 *E. coli* OP50-1 plates also lacking glucose, as well as paraquat, were sealed with parafilm. 15 hours after animals were introduced onto the plates, 30-40 individuals per condition were immobilised, and images were taken at 32 x magnification. Fluorescence was detected with a GFP filter at 400 millisecond exposure, and DIC images were taken in parallel.

Fluorescence measurements were taken using ImageJ software (V. 2.0.0-rc-38/1.50b). Corrected total worm fluorescence (arbitrary units, AU) was calculated as Integrated Density - (area of selected worm x mean background fluorescence of three readings). Nematode intensity values were thereby normalised to body size and background intensity. Data were then plotted as boxplots displaying median fluorescence, IQR and maximum/minimum values along with outliers.

#### 3.3.6.4 Thrashing assay

*C. elegans* were synchronized and introduced onto the respective diets as described. At the indicated timepoints, 15 animals were picked onto an empty NGM plate and left for one minute to move away from bacteria. Animals were then picked into another empty NGM plate filled with 5 ml M9 buffer, left for 30 seconds to acclimatize, and the number of body bends per minute was counted (full bend to one side). The assay was repeated in two independent trials (30 animals total).

### 3.3.7 Microscopy

Lifespan and thrashing measurements were made with an Olympus stereomicroscope. Fluorescent reporter expression was observed, and images were taken, using a Carl Zeiss Axio Zoom V16 Fluorescence zoom microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

### 3.3.8 Statistical analysis

Statistical analysis of lifespan data (log rank test) was conducted using SigmaPlot software (v11.0; Systat Software, Inc) and R software (version 3.1.1) on Kaplan-Meier survival curves. The log-rank test was used to compare survival distributions between any two conditions in the experiment, giving *p* values and average survival times (Park *et al.*, 2017). The Holm-Sidak method was then applied for multiple correction (SigmaPlot software), if more than two groups were compared. All graphs, including Kaplan-Meier survival curves, were generated using R.

Data for stress reporter screens (*Phsp-6::gfp* and *Pgst-4*::*gfp*) were non-normal and skewed. Following log transformation (for *Pgst-4:gfp* expression) or square root transformation (for *Phsp-6::gfp* expression), the data fit a normal distribution and could therefore be analysed using parametric Welch ANOVA and Games-Howell *post-hoc* (a more powerful alternative to the non-parametric Kruskal-Wallis test). Thrashing data were compared using Welch ANOVA with Games-Howell *post-hoc* test (day 1) or One-way ANOVA with Tukey's *post-hoc* test (days 4, 7 and 10).

A summary of the hypotheses that were tested and the conclusions reached is given in Table 3. 1.

**Table 3. 1 Summary of conditions for the experiments in Chapter 3.** Where *B. s.* Δ*atp* was not concentrated, 150 µl (50 µl re-seeded x3) were seeded onto NGM(-Glu) to provide sufficient food. 50 µl of all other bacterial strains were seeded onto NGM(-Glu) or (+Glu) plates (whether concentrated or un-concentrated). unconc, unconcentrated bacteria; conc, concentrated bacteria.

| Fig. | Hypothesis / question | Bacterial strains | *C. elegans* culture conditions | | Growth * | Conclusion |
|---|---|---|---|---|---|---|
| | | | L1-L4 | Adulthood | | |
| 3.2 | Is post-embryonic development affected by *B. s.* Δ*atp*? | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: unconc, conc.<br>+Glu. | - | 24 hrs | *B. s.* Δ*atp* delays post-embryonic development regardless of food quantity |
| 3.3A | *B. s.* Δ*atp*(+Glu) protects *C. elegans* against the toxicity of glucose.<br><br>*B. s.* Δ*atp* only extends lifespan when glucose is present throughout larval development. | *B. s.* MS<br>*B. s.* Δ*atp* | +Glu. | +Glu. | 24 hrs | *B. s.* Δ*atp*(+Glu) extends lifespan beyond that in the absence of glucose, instead of protecting from glucose.<br><br>Larval development +Glu is not required for *B. s.* Δ*atp*(+Glu) to extend lifespan. |
| 3.3B | | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: MS unconc, Δ*atp* conc. | -Glu: MS unconc, Δ*atp* conc.<br><br>+Glu. | 24 hrs | |
| 3.4 | Concentrating *B. s.* Δ*atp*(-Glu) prevents it from extending lifespan. | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: conc. | -Glu: unconc.<br><br>+Glu. | 24 hrs | Lifespan is unaffected by *B. s.* Δ*atp*(-Glu) whether bacteria are concentrated or unconcentrated. |
| 3.5 | Deletion of *E. coli* ATP synthase mirrors the effect of *B. s.* Δ*atp* in the presence of glucose. | *E. coli* DK8<br>*E. coli* DK8 Δ*unc* | -Glu: conc. | -Glu and +Glu: conc. | 24 hrs | *E. coli* lacking ATP synthase does not disproportionately extend lifespan in the presence of glucose. |
| 3.6A | | *B. s.* MS | -Glu: unconc. | -Glu: unconc +FUdR. | 48 hrs | |

| Fig. | Hypothesis / question | Bacterial strains | *C. elegans* culture conditions | | Growth * | Conclusion |
|---|---|---|---|---|---|---|
| | | | L1-L4 | Adulthood | | |
| | The *C. elegans* sterilizing chemical FUdR has no effect in the *B. subtilis* metabolism and is not an important variable in *C. elegans* lifespan assays. | *B. s.* Δ*atp* | +Glu. | +Glu: +FUdR. | | *B. s.* Δ*atp* extends *C. elegans* lifespan with and without glucose in the presence of FUdR.<br><br>FUdR dramatically influences bacterial growth and *C. elegans* lifespan. |
| 3.6B | | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: conc | -Glu: conc +FUdR.<br><br>+Glu: +FUdR. | 48 hrs | |
| 3.8 and 3.9 | Longevity of *B. s.* Δ*atp*(+Glu) arises through stress-induced hormesis.<br><br>Concentrating the bacteria does not influence stress responses. | *B. s.* MS<br>*B. s.* Δ*atp*<br>*E. coli* OP50-1 | -Glu: conc and unconc. | -Glu: conc and unconc.<br><br>+Glu: (from -Glu unconc). | 24 hrs | Stress-induced hormesis through $UPR^{mt}$ or cytosolic oxidative stress is unlikely to mediate longevity.<br><br>*C. elegans* fed on concentrated *B. s.* (-Glu) are undergoing oxidative stress. |
| 3.10 | Longevity of nematodes fed on *B. s.* Δ*atp* is associated with enhanced health. | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu, unconc. | -Glu.<br><br>+Glu. | 24 hrs | *B. s.* Δ*atp* extends the healthspan of *C. elegans* with and without glucose. |

* the length of time in which bacteria were left to grow on NGM prior to the introduction of nematodes at 20 °C.

### 3.4 Results

Preliminary data indicated that a *B. subtilis* mutant lacking ATP synthase (*B. s.* Δ*atp*) extended lifespan in the presence of glucose. This suggests that, through the action of an ATP synthase-deficient diet, glucose became beneficial to *C. elegans.* In this chapter, the effect of the mutant diet on post-embryonic development, lifespan and health was assessed.

#### 3.4.1 *B. s.* Δ*atp* induced a developmental delay in *C. elegans*

As described in Chapter 1, section 1.7, monitoring the expression of the dietary sensor *Pacdh-1::gfp* was conceived as a means to identify *B. subtilis* 168 deletion mutants that might influence *C. elegans* lifespan. Downregulation of this gene by *B. s.* Δ*atp*(+Glu) was confirmed in day 2 animals (Fig. 3. 1), as was observed in the preliminary screen. However, animals were also screened on each diet in the absence of exogenous glucose, and in this case reporter downregulation by *B. s.* Δ*atp* was even more dramatic. Notably, nematodes appeared to be smaller when fed on the mutant diet (body volume is quantified in Chapter 4).

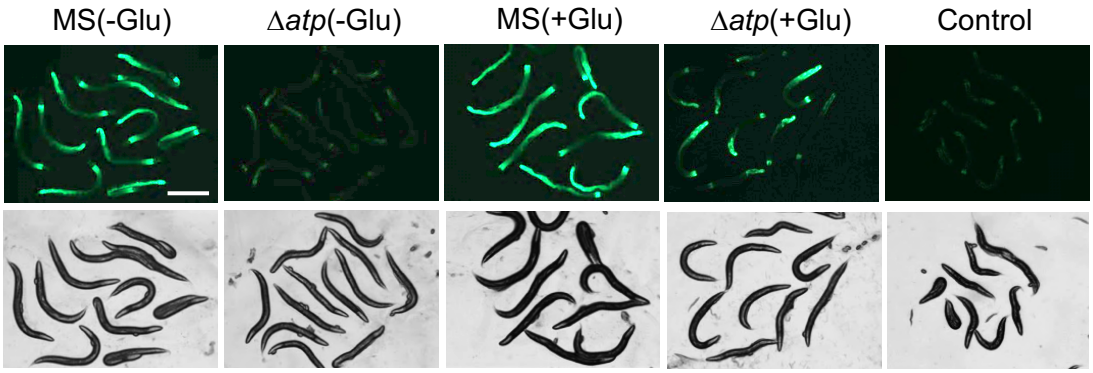



**Figure 3. 1 Expression of *Pacdh-1::gfp* was reduced in *C. elegans* fed on *B. subtilis* Δ*atp* in the absence (-Glu) and the presence (+Glu) of glucose.** Images were taken of day 2 adults to provide a visual screen for diet sensor expression. Lawns were unconcentrated on NGM(-Glu). Nematodes fed on glucose plates were introduced onto these plates 48 hrs previously. The starvation control nematodes lacked any bacteria to feed on from L4 until imaging. Images are representative of at least 30 individuals. Scale bar = 0.5 mm.

Dietary restriction (DR) can downregulate *acdh-1* expression (Van Gilst *et al.*, 2005). The development of *C. elegans* is sensitive to stressors and nutrient availability. Development of *C. elegans* was monitored on each of the *B. subtilis* diets. A delay in post-embryonic development was observed in nematodes that were fed on *B. s.* Δ*atp* (Fig. 3. 2), which generally lasted approximately three hours (not quantified), also consistent with DR. DR is commonly induced by limiting food availability to the nematodes. Surprisingly, however, the developmental delay tended to be more severe in the presence of glucose, where bacterial growth was abundant. Here, larvae were often markedly asynchronous at the timepoint at which *B. s.* MS-fed nematodes were at the late L4 stage, and a considerable number of L1 larvae were observed on lawns of *B. s.* Δ*atp*(+Glu) at this time point. This indicates that a proportion of the population had entered into L1 arrest. These observations will be further explored in Chapter 4 in relation to the formation of an extensive extracellular matrix by *B. s.* Δ*atp*(+Glu), which is hypothesised to restrict feeding.

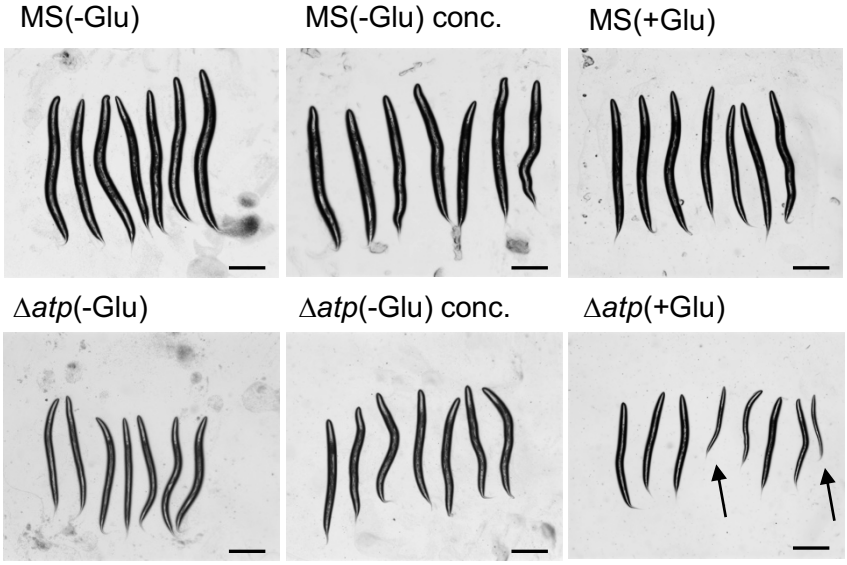



**Figure 3. 2 Development of *C. elegans* at 20 °C was delayed by a diet of Δ*atp*(-Glu) and Δ*atp*(+Glu).** All images were taken 48 hrs after introduction of L1s onto the indicated diet (50 x magnification). Images show a representative set of larvae from each of the diets. All nematodes fed on MS (top row) had entered the mid/late L4 stage by the time of imaging, whereas a majority of Δ*atp*-fed animals were L3 or early L4. Animals fed on Δ*atp*(+Glu) were markedly asynchronous, with a number of animals still in the L1 stage (arrows). Conc = concentrated bacterial lawn (see Materials and Methods). Scale bar = 200 μm.

To determine whether changes in food quantity or food composition were responsible for eliciting the developmental delay in animals fed on *B. s.* Δ*atp*(-Glu), the bacteria were concentrated 60x as described in Chapter 3 section 3.3. This ensured that *C. elegans* obtained sufficient food throughout the course of the experiment. As shown in Fig. 3. 2, concentrating the bacteria did not relieve the developmental delay of nematodes fed on *B. s.* Δ*atp*(-Glu). This demonstrates that the delay phenotype was not a result of low bacterial quantity, but of a change in bacterial composition compared to *B. s.* MS.

In order to ensure that *B. s.* Δ*atp* levels did not run too low without glucose, the bacteria were routinely concentrated in the following experiments (except where indicated).

### 3.4.2 Investigating the effect of *B. s.* MS and Δ*atp* on the lifespan of *C. elegans*

Here, the effect of changing certain variables which are likely to influence nematode lifespan was investigated. These variables were as follows:

1. The time point at which nematodes were exposed to glucose. As indicated, nematodes were introduced onto NGM(+Glu) plates as L1 larvae (referred to as L1+Glu) until death. Alternatively, nematodes were introduced onto NGM(+Glu) plates as L4 larvae, prior to which they were fed on the same diet -Glu.
2. The method for seeding the NGM(-Glu) plates. The bacteria were either seeded directly from an overnight culture (unconcentrated) or were concentrated (conc.) prior to seeding.
3. The presence or absence of FUdR in the NGM plates.

#### 3.4.2.1 Larval glucose exposure was not required for the longevity of *C. elegans* fed on *B. s.* Δ*atp*

The lifespan of *C. elegans* fed on *B. s.* MS and Δ*atp* in the presence and the absence of 2% glucose was measured. Although the subsequent adult lifespan of L1 arrested *C. elegans* is normal (Johnson *et al.*, 1984), exposure of young animals to non-lethal stressors can extend lifespan in a process called hormesis (Kishimoto *et al.*, 2017). The hypothesis that stress throughout larval development contributed to the longevity of animals fed on *B. s.* Δ*atp*(+Glu) was investigated. In Fig. 3. 3A, only longevity in the presence of glucose was analysed (Fig. 3. 3B). Here,

nematodes were introduced onto NGM(+Glu) plates from the L1 stage until death (L1+Glu), so that glucose was present throughout larval development. Under these conditions, nematodes fed on MS had a mean lifespan of 15.6 ± 0.5 days, with a maximum of 23.4 days (N = 100). Feeding on *B. s.* Δ*atp*, however, extended average lifespan by 52% (24.7 ± 0.56 days), and led to a maximum lifespan of 31.6 days (N = 102). The shape of the survival curves +Glu was significantly different ($p < 0.0001$, log rank test). See Appendix Table A1 for statistical analyses.

In Fig. 3. 3B, *C. elegans* were fed on NGM(+Glu) only as L4 larvae onwards. Lifespan assays were also conducted in the absence of glucose on unconcentrated lawns of *B. s.* MS and concentrated lawns of *B. s.* Δ*atp*. *B. s.* Δ*atp*(+Glu) was still able to extend nematode lifespan, even when animals were only introduced onto NGM(+Glu) plates from L4. The mean lifespan of nematodes fed *B. s.* Δ*atp*(+Glu) was 23.6 ± 0.66 days (N = 122) compared to 19.3 ± 0.36 days (N = 131) for those fed MS(+Glu). The shape of the curves was significantly different ($p < 0.0001$, log rank test). However, the magnitude of the lifespan extension induced by *B. s.* Δ*atp* was dramatically lessened when larval development occurred in the absence of glucose (22%, Fig. 3. 3B vs. 52%, Fig. 3. 3A). Interestingly, comparing the curves in Fig. 3. 3, there was no difference between the lifespan of animals fed on *B. s.* Δ*atp*(L1+Glu), and those fed in the absence of glucose as larvae ($p = 0.633$, log rank test). Instead, larval exposure to glucose reduced the lifespan of individuals fed on *B. subtilis* MS ($p < 0.0001$, log rank test). This shows that a diet of *B. s.* Δ*atp*(+Glu) during adulthood alone was sufficient to extend lifespan.

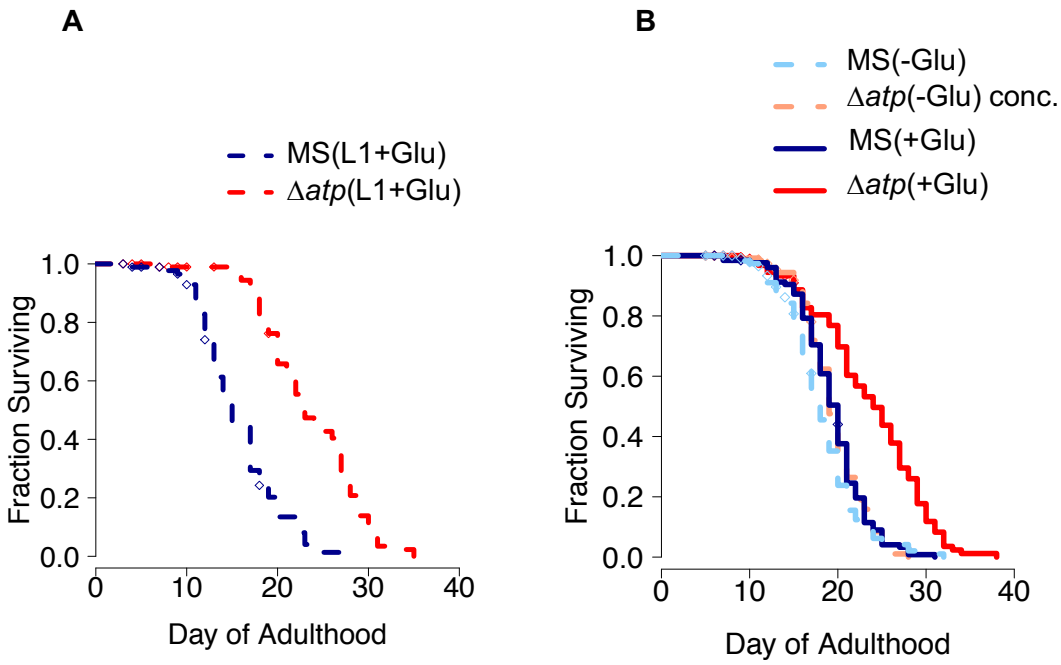



**Figure 3. 3 *B. subtilis* Δ*atp* extended the lifespan of *C. elegans* +Glu but not -Glu.** (A) Survival of *C. elegans* fed on *B. s.* MS or Δ*atp* in the presence of glucose from L1 until death (L1+Glu). Feeding on Δ*atp* increased mean lifespan by 52%. The difference in the survival curves is statistically significant ($p < 0.0001$). N=100-102 (B) Survival of *C. elegans* fed *B. subtilis* MS(+Glu) or Δ*atp*(+Glu) from mid-late L4 onwards, and -Glu. *B. s.* Δ*atp* lawns were concentrated (conc.) in the absence of glucose to provide sufficient food. There was no statistically significant difference in lifespan -Glu ($p = 0.2$). *B. s* Δ*atp*(+Glu) significantly increased nematode lifespan, compared to MS(+Glu)-fed individuals ($p < 0.0001$). N=121-131. All lifespan experiments were conducted at 20 °C. Diamond marks represent censoring. Log rank test with Holm-Sidak test for multiple comparisons on Kaplan Meier survival curves.

Unexpectedly, as shown in Fig. 3. 3B, glucose did not reduce the lifespan of individuals fed *B. s.* MS, compared to MS(-Glu)-fed animals ($p = 0.2$, log rank test). This indicates that glucose was not toxic to *C. elegans.* Moreover, *B. s.* Δ*atp*(-Glu) (N = 121) did not extend lifespan compared to *B. s.* MS(-Glu) (N = 121) ($p = 0.2$, log rank test).

A diet of *B. s.* Δ*atp*(+Glu) stimulated higher levels of escape from the assay plates during the experiment shown in Fig. 3. 3B (Appendix Table A1). 30% censoring was recorded on this diet, primarily due to escape, compared with the escape of only a single animal fed on *B. s.* MS(+Glu). The level of censoring was lower, but still high, on *B. s.* Δ*atp*(-Glu) (21%) and was almost as high (18%) on *B. s.* MS(-Glu). In contrast, a more similar level of escape was recorded when animals were exposed to glucose through the whole of their lives (Fig. 3. 3A); 14.7% of

nematodes fed on *B. s.* Δ*atp*(L1+Glu) were censored, compared to 22% of worms fed on *B. s.* MS(L1+Glu), primarily as a result of escape in both cases.

In Fig. 3. 3B, only *B. s.* Δ*atp*(-Glu) was concentrated. To control for the effect of concentrating the lawn, the experiment was repeated with concentrated lawns of both diets. *B. s.* MS was concentrated an equivalent amount based on $OD_{600}$ readings of overnight cultures (see Appendix Fig. A1). Here, again, there was no difference in lifespan ($p = 0.42$).

One noteworthy observation is the high frequency of vulval integrity defects (vulval explosion) in animals fed on *B. s.* MS(-Glu) and ∆*atp*(-Glu) (~16% in both cases compared to below 2% with glucose) (see Appendix Table A1). These animals were censored, as they usually are in lifespan analyses. When *B. s* MS(-Glu) as well as ∆*atp*(-Glu) was concentrated (Appendix Fig. A1), the frequency of vulval explosion was particularly high in animals fed on ∆*atp*(-Glu) (13%) in comparison to those fed on MS(-Glu) (2%) (see Table A1).

These experiments demonstrate that 1) larval development need not occur in the presence of glucose for *B. s.* ∆*atp* to extend lifespan, and 2) *B. s.* ∆*atp*(+Glu) promotes lifespan extension beyond that of animals fed -Glu, rather than simply protecting against the negative effects of glucose.

#### 3.4.2.2 Lifespan was not extended by lawns of unconcentrated *B. s.* Δ*atp*(-Glu)

Concentrated bacteria were previously provided in order to ensure a sufficient source of *B. s.* Δ*atp* for *C. elegans.* However, it was not clear whether this was necessary to avoid diet-restricting *C. elegans.* In order to investigate this, lifespans without glucose were measured without concentrating the bacteria prior to seeding by another experimenter (Neilson, 2017). The absence of a lifespan extension would indicate that DR is not induced. In parallel, lifespan was again measured in the presence of glucose (Fig. 3. 4). It is important to note, however, that from L1 until L4 all nematodes were fed on concentrated bacteria without glucose. As L4s, they were then transferred either onto unconcentrated lawns -Glu, or onto NGM(+Glu) plates.

Again, the lifespan of *C. elegans* was extended by *B. subtilis* lacking ATP synthase only when glucose was present ($p < 0.0001$, log rank test, N = 76-105).

This time, the magnitude of longevity was even higher (42% increase in mean lifespan). In the absence of glucose, lifespan was unaffected ($p$ = 0.178, log rank test). The results corroborated the findings presented in Fig. 3. 4. Moreover, *B. s.* Δ*atp* still failed to increase lifespan without glucose even when the bacteria were not concentrated throughout adulthood, indicating that DR was not being elicited. Glucose also increased the lifespan of animals fed on *B. s.* MS, although to a much smaller extent (4%>, $p$ < 0.01).

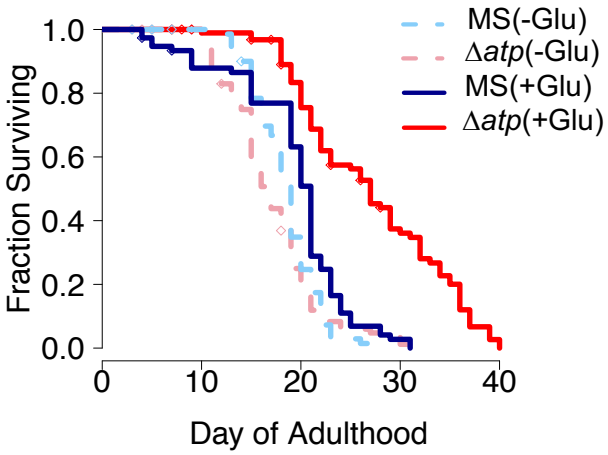



**Figure 3. 4 Unconcentrated *B. subtilis* Δ*atp*(-Glu) did not extend *C. elegans* lifespan.** A diet of Δ*atp* extended *C. elegans* lifespan +Glu ($p$ < 0.0001) but not -Glu ($p$ = 0.178), in comparison to animals fed on MS(+Glu) and MS(-Glu), respectively. Here, bacteria were not concentrated prior to seeding onto NGM(-Glu) except during development. N=76-106. The experiment was conducted at 20 °C. Diamond marks represent censoring. The log rank test with Holm-Sidak test for multiple comparisons was used for pairwise comparisons between conditions. Data were collected by Neilson (2017).

### 3.4.2.3 *B. subtilis* and *E. coli* mutants of ATP synthase had contrasting effects on lifespan +Glu or -Glu

Previous reports using *E. coli* lacking in ATP synthase show that these mutants extend *C. elegans* lifespan, without glucose being present in the media. However, lifespan of *C. elegans* had not previously been measured on these strains in the presence of glucose. To test the hypothesis that ATP synthase deficiency in *E. coli* promotes longevity to a greater extent in the presence of glucose, *C. elegans* were fed on ATP synthase-competent and ATP synthase-deficient strains of this species (DK8 and DK8 Δ*unc*, respectively). The experiment was conducted by Neilson (2017). Like *B. s.* Δ*atp, E. coli* strain DK8 Δ*unc* lacks the entire ATP

synthase operon. Results of the lifespan assays are given in Fig. 3. 5 (see Appendix Table A2 for statistical analyses).

DK8 Δ*unc*(-Glu) extended the mean lifespan of *C. elegans* (21.10 ± 0.54, N = 120) in comparison to nematodes fed on ATP synthase-competent DK8(-Glu) (16.92 ± 0.49, N=120) by 25% ($p < 0.0001$, log rank test). When glucose was present, DK8 Δ*unc* again extended lifespan ($p = 0.0027$, log rank test, N=111-118). However, the increase in mean lifespan was smaller than when glucose was absent (13%). In contrast to nematodes fed on *B. s.* MS, therefore, glucose abolished the majority of the longevity afforded by a diet of respiration-deficient bacteria. The presence of glucose reduced the lifespan of *C. elegans* fed on both DK8 ($p < 0.0001$, log rank test, 23% reduction in mean lifespan) and DK8 Δ*unc* ($p < 0.0001$, log rank test, 30% reduction in mean lifespan). This shows that extended longevity in the presence of glucose is not a general phenomenon of *C. elegans* fed on ATP synthase-deficient bacteria but arises from some particular aspect of *B. subtilis* metabolism.

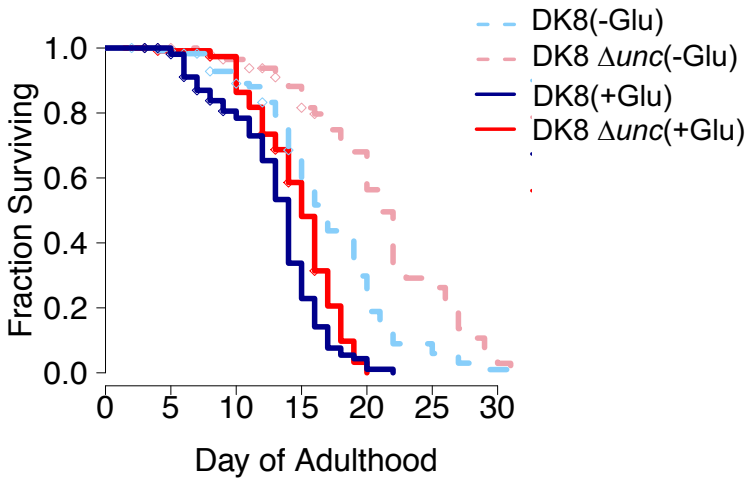



**Figure 3. 5 Survival of *C. elegans* fed on *E. coli* strain DK8 or DK8 Δ*unc*.** Lifespan was extended by DK8 Δ*unc* (lacking ATP synthase) -Glu ($p$ <0.0001) and +Glu ($p < 0.01$). N=111-120. All lifespan experiments were conducted at 20 °C. Diamond marks represent censoring. Log rank test with Holm-Sidak test for multiple comparisons on Kaplan Meier survival curves. Data were collected by Neilson (2017).

#### 3.4.2.4 *B. s.* Δ*atp* extended lifespan in the presence of 5-Fluoro-2'-deoxyuridine (FUdR)

The chemical 5-Fluoro-2'-deoxyuridine (FuDR), a DNA synthesis inhibitor that prevents germ cell proliferation and therefore the hatching of eggs, is frequently employed when measuring *C. elegans* lifespan. As it prevents progeny growth, FUdR removes the requirement for frequent (daily or every other day) transfer of the individuals under investigation as well as eliminating the internal hatching of larvae (bagging). 100 μM FUdR was added to the NGM(-Glu) and NGM(+Glu) plates. Despite being typically viewed as a benign variable, there is evidence that the inclusion of FUdR alters the lifespan of mutant *C. elegans* strains (Aitlhadj and Sturzenbaum, 2010; Rooney *et al.*, 2014). In one case, FUdR promoted longevity in a mutant strain without affecting wild-type lifespan (Van Raamsdonk and Hekimi, 2011). Moreover, bacterial metabolism mediates the effect of FUdR on *C. elegans* fecundity, and its effects are altered by different diets (García-González *et al.*, 2017). Here, the hypothesis that FUdR does not alter the metabolism of *B. subtilis,* and does not affect *C. elegans* lifespan, was tested.

Two variations on this experiment were carried out. In the first experiment (Fig. 3. 6A), the L1 larvae were introduced onto NGM(-Glu) or NGM(+Glu) plates and maintained on the same condition until death. Therefore, larvae developed in the presence of glucose for the +Glu conditions (L1+Glu). The bacterial lawns on NGM(-Glu) plates were seeded directly from an overnight culture (unconcentrated). In (Fig. 3. 6B), the bacteria were concentrated (conc.) 60 x before seeding the plates (see Methods). Secondly, larvae were only introduced onto NGM plates containing glucose as mid/late L4 larvae, to avert the asynchrony associated with larval development on *B. s.* Δ*atp*(+Glu) plates. Prior to this, larval development occurred in the absence of glucose on the respective diets.

In both experiments, *B. s.* Δ*atp* significantly extended *C. elegans* lifespan compared to MS in the presence and absence of glucose. A diet of *B. s.* Δ*atp*(-Glu) increased lifespan compared to *B. s.* MS(-Glu)-fed individuals whether the lawn was unconcentrated (Fig 3. 6A) or concentrated (Fig 3. 6B) (an increase of 22% and 35%, $p = 0.0153$, $p < 0.0001$ respectively, log rank test). *B. s.* Δ*atp* was therefore able to promote longevity in the absence of glucose when FUdR was present, in contrast to what was observed in the absence of FUdR.

When nematodes were exposed to glucose throughout the whole of their lives (Fig 3. 6A), *B. s.* Δ*atp*-fed individuals (N=117 minus censoring) had a mean lifespan that was 23% and 28% longer than those fed Δ*atp*(-Glu) (N=121) and MS(L1+Glu) (N=120), respectively. The difference in survival was statistically significant ($p < 0.0001$, log rank test). 2% glucose similarly extended the mean lifespan of nematodes fed on MS by 17%. Again, the difference in the shape of the distributions was significant ($p = 0.0017$, log rank test). In contrast, when nematodes were exposed to glucose from L4 onwards (Fig. 3. 6B), glucose appeared to be toxic to nematodes fed on both diets. A comparison of Fig 3. 6A and 3. 6B would therefore suggest that glucose during larval stages L1-L4 was beneficial. 2% glucose reduced mean nematode lifespan by 10% and 19% when fed on *B. s.* MS (N=124) and Δ*atp* (N=121), respectively, compared to the same diet in the absence of glucose (Fig. 3. 6B) ($p = 0.0002$, $p = 0.0016$ respectively, log rank test). A summary of the lifespan assays with FUdR is given in Appendix Table A3.

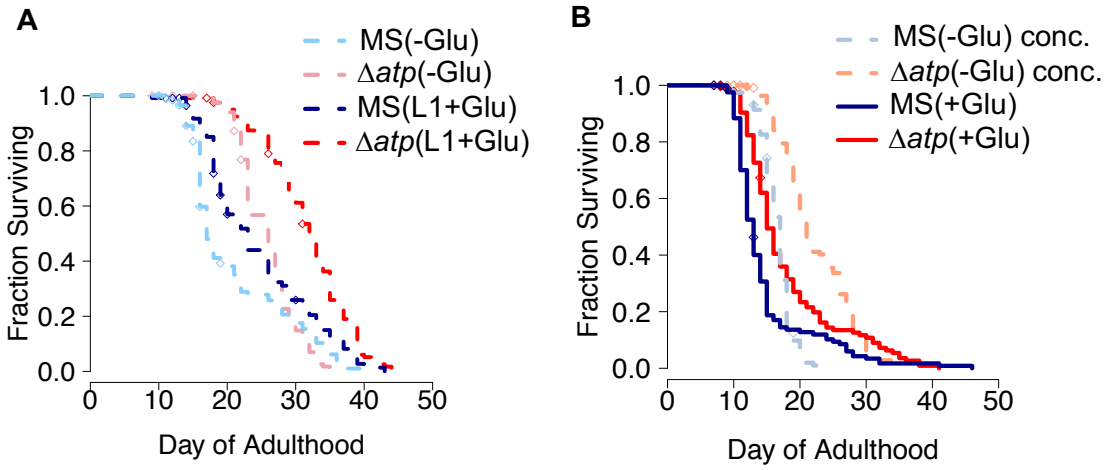



**Figure 3. 6 *B. subtilis* Δ*atp* extended lifespan of *C. elegans* in the presence of FUdR.** (A) Survival of *C. elegans* fed -Glu, or +Glu from the first larval stage until death (L1+Glu). The mean lifespan of nematodes fed Δ*atp*(+Glu) (N=121) was 28% longer than nematodes fed on MS(+Glu) (N=120). *B. subtilis* Δ*atp* significantly increased lifespan +Glu ($p < 0.001$). Even -Glu, *B. subtilis* Δ*atp* extended lifespan compared to MS ($p = 0.0152$). Glucose significantly increased the lifespan of MS-fed individuals ($p = 0.0017$). (B) Survival of *C. elegans* fed on concentrated (conc) lawns -Glu, or +Glu only from mid/late L4 onwards. *B. subtilis* Δ*atp* extended lifespan both with ($p = 0.0002$) and without ($p < 0.0001$) glucose. In contrast to (A), glucose reduced the lifespan of individuals fed on both *B. s.* MS ($p = 0.0002$) and *B. s.* Δ*atp* ($p = 0.0016$). All lifespan experiments were conducted at 20 °C. Diamond marks represent censoring. Log rank test with Holm-Sidak test for multiple comparisons on Kaplan Meier survival curves.

Strikingly, the curves in Fig. 3. 6B show several unusual features. The maximum lifespans were reduced in comparison to Fig 3. 6A. The mean lifespans +Glu were even more dramatically reduced, giving the curves a prolonged tail and causing the curves to cross. This partly arose from high rates of early-life mortality, which are apparent from the steep slope of the curves around day 10-20 of adulthood. Additionally, the curve for nematodes fed *B. s.* MS(-Glu) in Fig 3. 6B is stunted, with a maximum lifespan of only 20 days. The reason for this frequent early death/censoring is unclear. Analysis of the shape of the curves suggests that, although glucose increased the initial mortality risk, long-term survival was improved when glucose was present.

The bacterial diet of *C. elegans* mediates the effects of FUdR on fecundity and lifespan (García-González *et al.*, 2017). Metabolism of both *B. subtilis* strains was altered by this chemical, as shown by a clear shift in lawn growth and morphology (Fig. 3. 7). Lawns of both strains were looser and thinner in the presence of FUdR. This chemical prevented or slowed the formation of a visible structure resembling extracellular matrix, which was apparent when FUdR was absent.

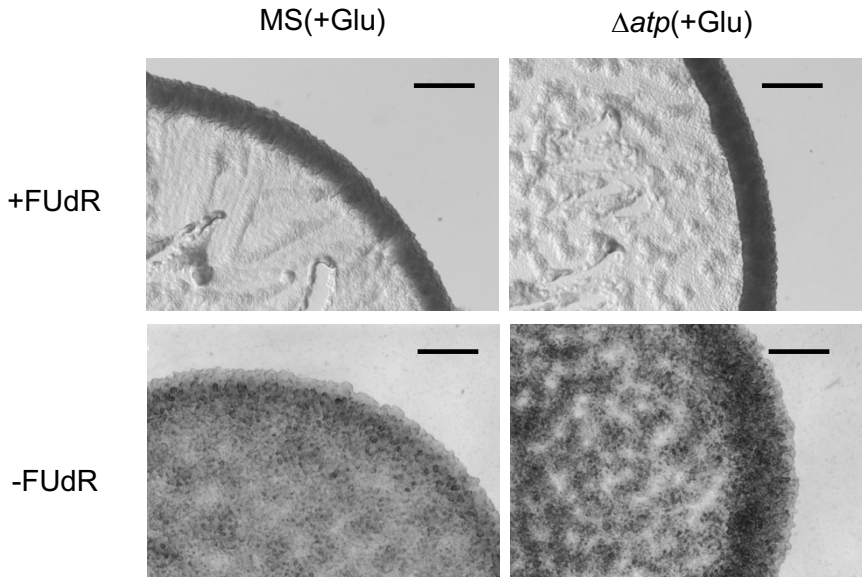



**Figure 3. 7 FUdR altered bacterial lawn morphology.** When grown on NGM(+Glu) lacking FUdR (bottom row), *B. s.* MS and Δ*atp* developed an extracellular matrix which was absent in the presence of FUdR (top row). All lawns were grown in the presence of glucose for 36 hrs at 20 °C before imaging. Scale bar = 1 mm.

The presence of FUdR therefore had a considerable effect on the metabolism of *B. subtilis,* which was able to affect *C. elegans* lifespan. As a result, it was excluded from subsequent analysis.

### 3.4.3 Stress reporter expression was low in *C. elegans* fed on *B. subtilis* Δ*atp*(+Glu)

Stress responses are closely connected to lifespan and health. Low-level induction of stress responses is potentially able to hormetically extend lifespan in *C. elegans* and other organisms, whilst extended activation of the same pathways can suppress longevity (Tauffenberger *et al.*, 2016). The expression of detoxification and antioxidant genes is commonly observed in *C. elegans* and other organisms under conditions of nutrient deprivation or starvation (Tao *et al.*, 2017), which can increase lifespan. In contrast, the presence of glucose can upregulate the expression of stress reporter genes in the nematode and compromise health (Tauffenberger *et al.*, 2016; Zhu *et al.*, 2016). The effect of *B. subtilis* Δ*atp*(+Glu) on *C. elegans* stress responses was investigated.

The involvement of two major stress pathways, the cytosolic oxidative stress response and the mitochondrial unfolded protein response ($UPR^{mt}$), were screened using reporter strains for *gst-4* and *hsp-6* expression, respectively. *hsp-6* encodes a mitochondrial chaperone which responds to mitochondrial stress or dysfunction. HSP-6 is an orthologue of the mammalian mitochondrial heat shock protein 70, which regulates lifespan and cell proliferation in mammalian cells (Kimura *et al.*, 2007). Up-regulation of *hsp-6* is associated with induction of the $UPR^{mt}$, which entails the expression of nuclear-encoded mitochondrial chaperones in response to misfolding of mitochondrial proteins, amongst other stresses (Bennett and Kaeberlein, 2014). Glutathione S-transferases are enzymes which detoxify harmful compounds including the products of oxidative stress, through the conjugation of glutathione to the target (Salinas and Wong, 1999). *gst-4* plays an important role in the response of *C. elegans* to oxidative stress; *gst-4* overexpression can enhance the resistance of *C. elegans* to oxidative stress, whilst knockdown of this gene using RNAi compromises oxidative stress resistance (Ayyadevara *et al.*, 2005; Leiers *et al.*, 2003). *Phsp-6::gfp* and *Pgst-4::gfp* reporter strains were fed on *B. subtilis* MS and Δ*atp,* and fluorescence intensity of day one adults was quantified (Fig. 3. 8 and Fig. 3. 9). Expression levels in nematodes fed *E. coli* OP50-1 were also investigated, as reporter expression is well characterised in *C. elegans* fed on this

diet. Paraquat has been shown to induce expression of *Pgst-4::gfp* (Tawe *et al.*, 1998) and *Phsp-6::gfp* (Runkel *et al.*, 2013), and was therefore used as a positive control for both stress reporters. See Appendix Table A4-A7 for summary statistics and comparisons.

Welch ANOVA omnibus tests revealed a significant effect of diet on the expression of *Phsp-6::gfp* ($F(8,121) = 110.8435$, $p < 0.0001$) and *Pgst-4::gfp* ($F(8, 122) = 76.4731$, $p < 0.0001$). Pairwise comparisons were then made on both datasets using the Games-Howell *post-hoc* test. *B. subtilis* Δ*atp* did not induce *Phsp-6*::*gfp* expression in either the absence or the presence of glucose (Fig. 3. 8). The expression level of *B. s.* Δ*atp*(-Glu)-fed individuals was indistinguishable from that of nematodes fed *B. s.* MS(-Glu) or *E. coli*(-Glu) ($p > 0.05$). *B. s* Δ*atp*(+Glu) actually reduced the expression of this gene, in comparison to *B. s* MS(+Glu) and *E. coli*(+Glu) ($p < 0.0001$). In fact, nematodes fed on Δ*atp*(+Glu) had the lowest level of *Phsp-6::gfp* expression compared to all other conditions, significantly lower than that of Δ*atp*(-Glu)-fed individuals ($p < 0.0001$). Interestingly, concentrated *B. subtilis* MS(-Glu) and Δ*atp*(-Glu) induced high expression of the *Phsp-6*::*gfp* reporter, similar to the paraquat control, suggesting that nematodes were undergoing mitochondrial stress on these plates. Concentrating the bacteria might have unintended effects on the nematodes, calling into question the suitability of this method for feeding *C. elegans*.

The influence of bacterial diet on *Pgst-4::gfp* expression is given in Fig. 3. 9. Here, once again there was no difference in reporter expression on the two diets when the bacteria were concentrated ($p = 0.875$). Concentrating the *B. s.* Δ*atp*(-Glu) lawn did not change reporter expression compared to unconcentrated lawns ($p = 0.931$). However, concentrating MS(-Glu) lawns reduced reporter expression significantly compared to MS(-Glu) unconcentrated lawns ($p = 0.0005$). Reporter expression in nematodes fed on the *B. subtilis* diets without glucose was no different to those fed *E. coli*(-Glu) ($p > 0.05$). On unconcentrated lawns, *B. s.* MS up-regulated *Pgst-4::gfp* to a greater extent than *B. s.* Δ*atp* ($p = 0.0003$). This was even more dramatic +Glu ($p < 0.0001$) and appears to reflect up-regulation of reporter expression beyond the level of the paraquat-treated cohort in *B. s.* MS-fed individuals. This indicates that *B. s.* MS(+Glu) induced considerable cytosolic oxidative stress in *C. elegans*.

Both *E. coli*- and *B. s.* MS-fed nematodes had higher expression of *Pgst-4::gfp* when glucose was present, compared to when it was absent. This is consistent with a previous report, in which induction of *Pgst-4::gfp* in the presence of 200 mM glucose was observed in liquid medium (Zhu *et al.*, 2016). However, when nematodes were fed on *B. s.* Δ*atp,* glucose failed to induce *Pgst-4*::*gfp* ($p > 0.05$).

To summarise, feeding on *B. subtilis* Δ*atp*(+Glu) was associated with low-level expression of both *Pgst-4::gfp* and *Phsp-6*::*gfp*. This suggests that nematodes fed on Δ*atp*(+Glu) had low activation of the $UPR^{mt}$ and low levels of cytosolic oxidative stress and argues against the hypothesis that hormesis during adulthood elicits longevity in these individuals. Instead, animals fed on this diet appeared to be less stressed, or possibly less able to mount stress responses.

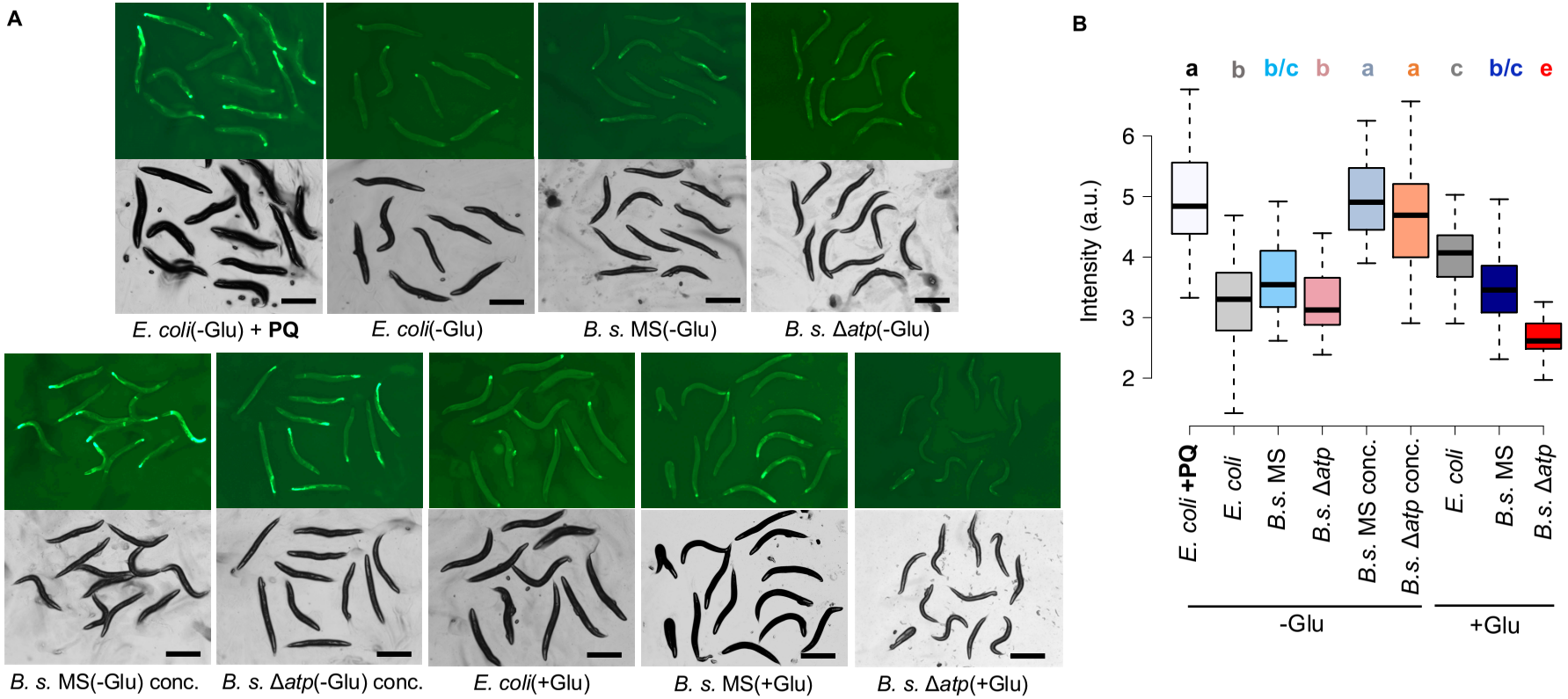



**Figure 3. 8 The expression of *Phsp-6::gfp*, a reporter for the UPR$^{mt}$, is low in animals fed on *B. s.* Δ*atp*(+Glu).** (A) Representative images of *C. elegans* expressing *Phsp-6::gfp* on each of the indicated diets. (B) Quantification of GFP expression on the indicated diets. Day 1 adult animals were screened. The development of all individuals (L1-L4) occurred in the absence of glucose. The effect of concentrating (conc.) the *B. s.* lawns -Glu was also investigated. *E. coli* refers to strain OP50-1. Positive control animals were fed on *E. coli* and were exposed to 200 mM paraquat (PQ) only in the absence of glucose for 15 hrs prior to screening to induce *hsp-6* expression. 30-40 animals per group were quantified. Different letters indicate significant differences between conditions ($p < 0.05$, Games-Howell *post-hoc* test following Welch ANOVA). A square-root transformation was applied to the data prior to statistical analysis to approximate a normal distribution. Scale bar = 0.5 mm.

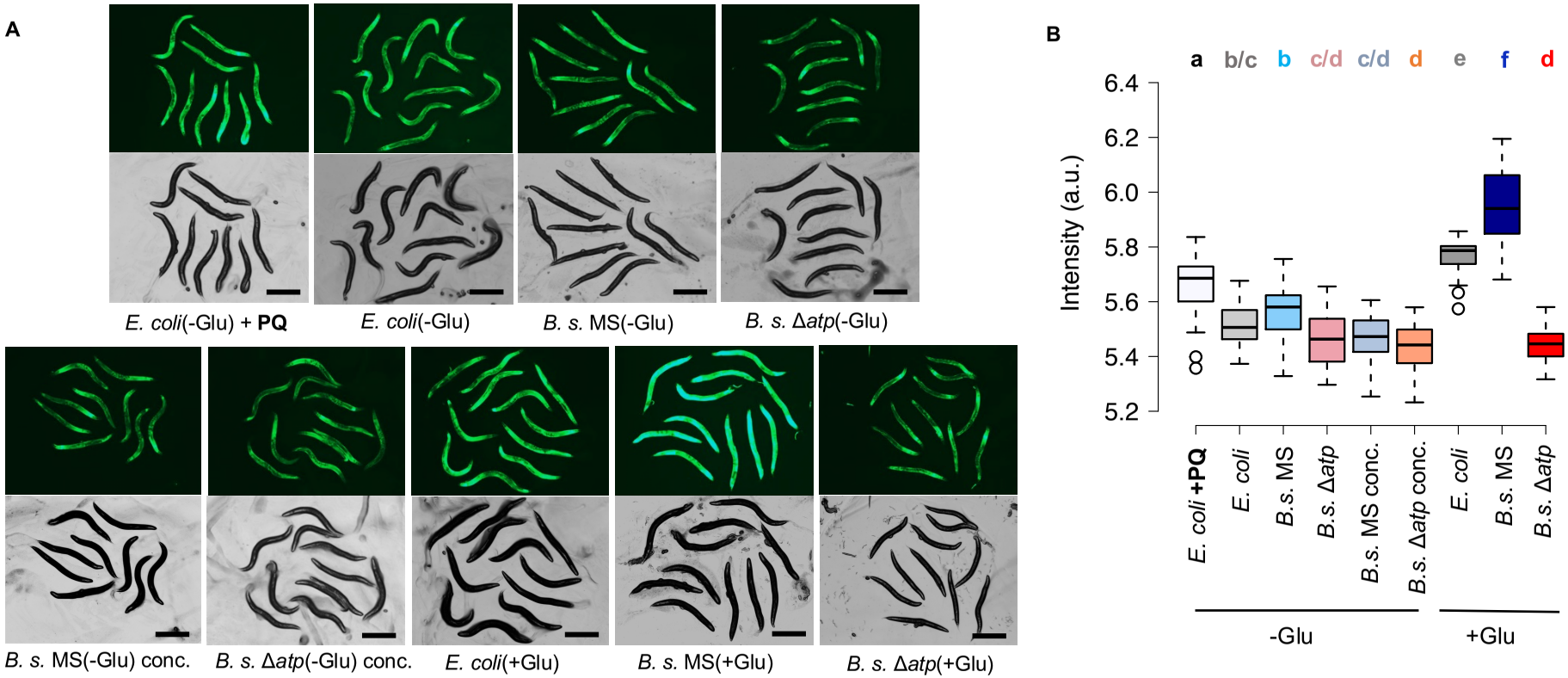



**Figure 3. 9 Expression of *Pgst-4*::*gfp*, a marker for oxidative stress, in response to glucose is high in nematodes fed on *B. s.* MS but low in those fed on *B. s.* Δ*atp*.** (A) Representative images of *C. elegans* expressing *Pgst-4::gfp* on each of the indicated diets. (B) Quantification of GFP expression on the indicated diets. Day 1 adult animals were screened. The development of all individuals (L1-L4) occurred in the absence of glucose. The effect of concentrating (conc.) the *B .s.* lawns in the absence of glucose was also investigated. *E. coli* refers to strain OP50-1. Positive control animals were fed on *E. coli*(-Glu) and exposed to 200 mM paraquat (PQ) for 15 hrs prior to screening. 30-40 animals per group were quantified. Different letters indicate significant differences between conditions ($p < 0.05$, Games-Howell *post-hoc* test following Welch ANOVA). Circles depict outliers. Data were log transformed prior to statistical analysis to approximate a normal distribution. Scale bar = 0.5 mm .

### 3.4.4 *B. s.* Δ*atp* preserved nematode motility with age

Extended lifespan is only desirable if it is concurrent with improved health (i.e. extended healthspan). Ageing *C. elegans* exhibit a decline in locomotion on solid and liquid media which is correlated with the extent of muscle deterioration (Herndon *et al.*, 2002; Huang *et al.*, 2004). In order to investigate whether nematodes fed on a diet of *B. s.* Δ*atp*(+Glu) were healthier than individuals provided with the other diets, motility was monitored in young adult and middle-aged nematodes. *C. elegans* were raised on each of the four diets and the motility (full body bends $min^{-1}$) in liquid medium of a total of 30 individuals was scored on days 1, 4, 7 and 10 of adulthood. The results are given in Fig. 3. 10. A one-way ANOVA (with Welch's correction for unequal variances for day 1) revealed a significant association between diet and the rate of thrashing on day 1 ($F(3, 61) = 70.525$, $p < 0.0001$), day 4 ($F(3, 116) = 55.126$, $p < 0.0001$), day 7 ($F(3, 116) = 56.308$, $p < 0.0001$) and day 10 ($F(3, 116) = 87.868$, $p < 0.0001$) of adulthood. Following omnibus tests, pairwise comparisons between groups were made on each day using the Games-Howell *post-hoc* test (day 1) or Tukey's HSD *post-hoc* test (days 4, 7 and 10). Here, bacteria seeded onto NGM(-Glu) were unconcentrated for the entirety of the experiment. See Appendix Table A8 for statistical analyses.

On day 1 of adulthood, the mean thrashing rate ± standard deviation of individuals fed on *B. s.* MS(-Glu) (76.5 ± 5.734) was significantly lower than all other conditions ($p < 0.0001$). Surprisingly, nematodes fed on MS(+Glu) were significantly more motile in liquid (96.233 ± 8.744) than those fed on MS(-Glu) on the first day of adulthood. However, the presence of glucose did not significantly affect thrashing rate of MS-fed individuals on day 4 ($p = 0.255$), day 7 ($p = 0.998$) or day 10 ($p = 0.569$).

When -Glu conditions are considered, a diet of *B. s* Δ*atp* induced a significant increase in motility at each timepoint ($p < 0.0001$). The same was true +Glu ($p = 0.0035$, day 1; $p < 0.0001$, days 4, 7 and 10). When *C. elegans* was fed on *B. s* Δ*atp*(+Glu), a peak in thrashing rate was observed on day 1 (105.9 ± 11.695), which exceeded that seen in individuals fed on Δ*atp*(-Glu) (91.933 ± 13.014) ($p = 0.003$). However, by day 4 this advantage was no longer apparent ($p = 0.0874$), and the thrashing rate of nematodes fed on Δ*atp*(-Glu) on day 7 (99.033 ± 12.442) and day 10 (96.233 ± 10.526) exceeded that of those fed on Δ*atp*(+Glu) (89.2 ± 11.699 and 82.067 ± 9.131, $p = 0.0092$ and $p < 0.0001$, respectively). The

increased motility afforded by a diet of *B. s.* Δatp(-Glu) was dramatic. Compared to animals fed on *B. s.* MS(-Glu), mean thrashing rate was increased by 20%, 32%, 48% and 66% on day 1, 4, 7 and 10, respectively.

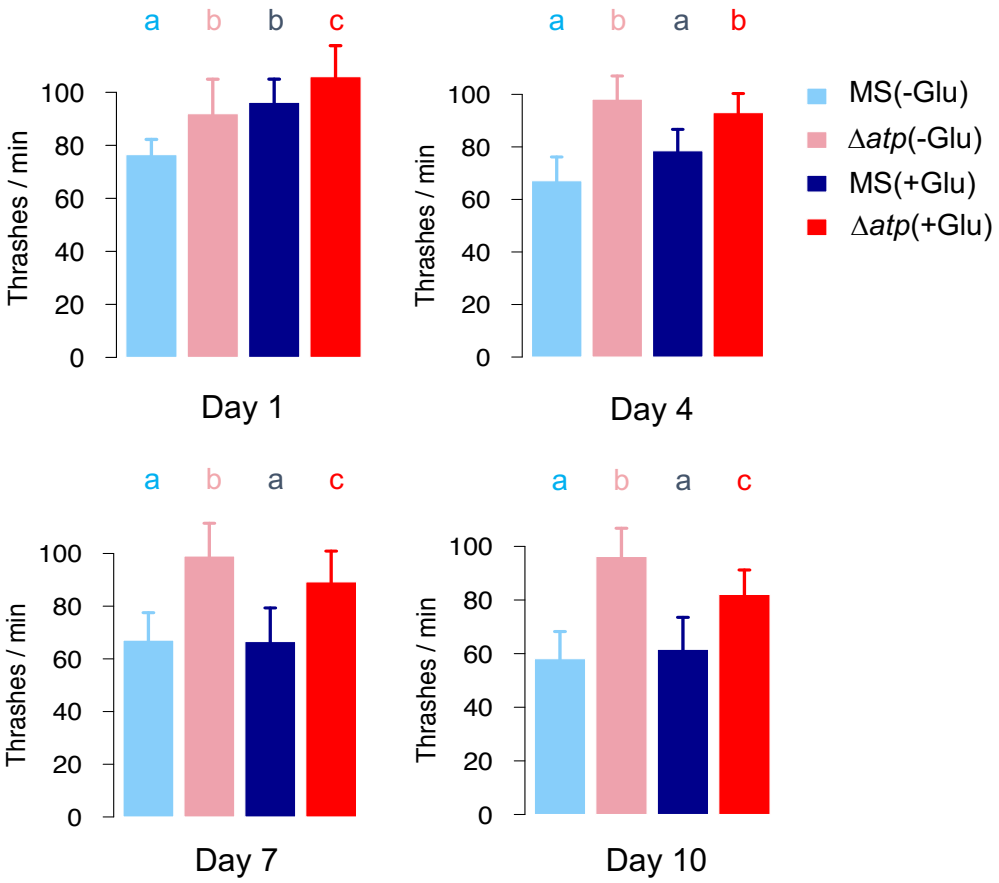



**Figure 3. 10 A diet of *B. subtilis* Δ*atp* slowed the age-related decline in motility -Glu and +Glu.** Thrashing rate (body bends / min in liquid media) showed little decline over a 10-day period in animals fed on *B. s.* Δ*atp*. On day 7 and 10, individuals fed on *B. s.* Δ*atp*(-Glu) were more motile than those fed on *B. s.* Δ*atp*(-Glu) ($p = 0.0092$, $p < 0.0001$, respectively). Different letters indicate a statistically significant difference in thrashing rate ($p < 0.05$). Values are the mean ± standard deviation of two replicates where 15 individuals were analysed per replicate (30 animals in total). Welch ANOVA with Games-Howell *post-hoc* test (day 1); One-way ANOVA with Tukey's *post-hoc* test (days 4, 7 and 10).

This experiment confirmed that animals fed on *B. s.* Δ*atp* had improved health, as measured by muscular function with age. Unexpectedly, this was true especially in the absence of glucose, under conditions very similar to those in which lifespan was not extended. This indicates that this diet uncouples longevity from improved health.

## 3.5 Discussion

### 3.5.1 *B. subtilis* Δ*atp*(+Glu) robustly extended the lifespan of *C. elegans*

The experiments in this chapter show that a diet of *B. s.* Δ*atp*(+Glu) was capable of extending the mean lifespan of *C. elegans* by up to 40%. No longevity was apparent without glucose. This data supports preliminary findings which indicated that *B. subtilis* lacking ATP synthase was able to act as a probiotic in the presence of glucose.

Experiments using *E. coli* have suggested that glucose is toxic to *C. elegans* even in low concentrations (Lee *et al.*, 2009; Schulz *et al.*, 2007). The lifespan assays in this chapter corroborate this, as the lifespan of *C. elegans* was reduced on a diet of *E. coli* DK8(+Glu). A previous report has indicated that *B. subtilis* with mutations in the *atp* operon consume twice as much glucose as the wild-type (Santana *et al.*, 1994). Is it possible that the longevity of *C. elegans* fed on *B. s.* Δ*atp* is a consequence of removal of glucose from the agar? The data presented here would suggest not, because *C. elegans* fed on *B. s.* Δ*atp*(+Glu) have a lifespan that is beyond that of animals in the absence of glucose. This demonstrates that this diet has an actively positive effect on lifespan.

Moreover, glucose consistently failed to reduce the lifespan of *C. elegans* fed on *B. s.* MS. This suggests that that glucose itself was not toxic to *C. elegans* fed on *B. subtilis*. The concentration of glucose used throughout, 2%, is relatively high in comparison to many published reports, and was sufficient to reduce the lifespan of *C. elegans* fed on the *E. coli* DK8 master strain and ATP synthase mutant (Fig. 3. 5). Moreover, in a previous report, the addition of 2% glucose into NGM, along with arginine, reduced 50% survival of *C. elegans* fed on *B. subtilis* 168 by 25% (Gusarov *et al.*, 2013). Therefore, glucose does not require *E. coli* to be toxic. The reason for the lack of glucose toxicity in animals fed on the *B. subtilis* 168 strain in this work demands further enquiry. One mundane explanation is, again, removal of glucose from the NGM. Given the high concentration of glucose used, and the fact that animals were transferred to fresh plates every other day, it would be surprising if *C. elegans* were not taking up some glucose from these plates. This is particularly true given that very low concentrations (0.01%) can reduce lifespan in animals fed on *E. coli* (Choi, 2011). Also arguing against this explanation, the lifespan of *C. elegans* fed on *B. s.* MS was actually slightly increased by glucose (4%>) in one of the lifespan experiments conducted (Fig. 3. 4). In order to determine

whether *C. elegans* was being exposed to glucose, the levels of internal glucose or glycogen in animals fed on these diets should be measured, as in Tauffenberger *et al.* (2012), and the level of glucose remaining in the NGM(+Glu) plates following two days of bacterial growth should also be determined.

Alternative explanations for the lack of glucose toxicity in the presence of *B. subtilis* can be postulated. Potentially, *B. s.* MS activates glucose-protective mechanisms. Indeed, various mechanisms capable of attenuating glucose toxicity have recently been identified. Enhanced activity of DAF-16, possibly through reduced IIS, is one possibility. Moreover, modulating the storage of glycogen, which accumulates on high-glucose diets, by interfering with glycogen synthase *gly-1* attenuates the lifespan-shortening and health-limiting effects of 2% glucose on *C. elegans* by promoting storage of glucose as trehalose (Seo *et al.*, 2018). Similarly, normal lifespan was restored in the presence of glucose by overexpressing glyoxylase-1 (Schlotterer *et al.*, 2009). As well as this, reducing the glucose-induced accumulation of saturated fatty acids by upregulating SREBP and MDT-15 was sufficient to prevent glucose from reducing lifespan (D. Lee *et al.*, 2015). Therefore, mechanisms including accumulation of trehalose through reduction in *gsy-1* function, reduced saturated fatty acid accumulation through higher activity of SREBP and MDT-15, and upregulation of glyoxylase-1 could conceivably offset glucose toxicity in animals fed on *B. s.* MS(+Glu). Measurement of internal fat levels in animals fed on the *B. subtilis* diets, as well as probing the activity of IIS, AMPK and autophagy-dependent effector mechanisms, can begin to provide insight into this.

### 3.5.2 Glucose was not beneficial to *C. elegans* fed on *E. coli* lacking ATP synthase

Regardless of whether *C. elegans* was taking in glucose, *B. s.* Δ*atp* proved beneficial to nematode lifespan in its presence. Strikingly, there was a fundamental difference in the effect of ATP synthase-deficient *B. subtilis* and *E. coli* DK8 on the lifespan of *C. elegans* +Glu. The presence of this sugar was highly beneficial in the presence of *B. subtilis,* but not *E. coli,* lacking ATP synthase. The mechanism behind the promotion of longevity by *B. subtilis* and *E. coli* lacking ATP synthase is therefore likely to be different. The data also do not undermine the hypothesis that changes in the growth, colonisation capacity and therefore pathogenicity of respiration-deficient *E. coli* are causal to its effects on lifespan. If *E. coli* lacking ATP

synthase had elicited longevity particularly +Glu, the pathogenicity hypothesis would have become more difficult to support, since increasing *E. coli* proliferation would have been beneficial. Instead, animals fed on the least proliferative bacteria (*E. coli* DK8 Δ*unc*(-Glu)) lived the longest, while those fed on the most proliferative strain (*E. coli* DK8 +Glu) were the shortest-lived. This implies that the toxicity of glucose is a consequence not only of direct toxicity but also the glucose-stimulated proliferation of *E. coli,* which is reduced in the ATP synthase mutant. Alternatively, some growth-independent aspect of bacterial metabolism that is toxic to *C. elegans* might be reduced in the ATP synthase mutant.

These observations are therefore consistent with the idea that the growth of the *E. coli* strains was largely causal to their effects on *C. elegans* lifespan. Whether bacterial load in the intestine is important for this effect is unclear and is an area of controversy as previously discussed. Alternatively, the release of *E. coli*-negative factors including lipopolysaccharide and coQ, or otherwise toxic activities that have been implicated in previous studies (Virk *et al.*, 2016), might link increased proliferation to *C. elegans* mortality. In contrast, the growth rate of the *B. subtilis* diets did not correlate with their effect on the lifespan of *C. elegans,* whether one considers proliferation to be beneficial or detrimental. Some aspect of *B. subtilis* metabolism in the presence of glucose that is not shared with *E. coli* therefore promotes nematode longevity.

### 3.5.3 Larval exposure to glucose was not required for longevity

*C. elegans* fed on *B. s.* Δ*atp*(+Glu) were frequently found in L1 arrest. Larval arrest can be triggered by stresses including high temperature, anoxia, ethanol and food shortage (Baugh, 2013). The primary signal for L1 arrest is food shortage, and the potential role of this stress in mutant-induced longevity will be explored in Chapter 4. The results in this chapter show that the heightened larval stress experienced by *C. elegans* fed on *B. s.* Δ*atp*(+Glu) was not required for the longevity of these individuals. *B. s.* Δ*atp* was capable of extending the lifespan of *C. elegans* regardless of whether post-embryonic development occurred in the absence or in the presence of glucose, as long as glucose was present throughout adulthood. The mean lifespan of *C. elegans* was very similar in each case. In many cases, developmental delay and lifespan are coupled; for example, in feeding-restricted animals which have extended lifespan, or in *C. elegans* fed on *Comamonas* which have accelerated development but reduced lifespan (MacNeil *et*

*al.*, 2013). However, the induction of a developmental delay by *B. s.* Δ*atp* even -Glu demonstrates that delayed post-embryonic development does not necessarily lead to extension of lifespan.

Interestingly, a previous study reported that *E. coli* with mutations in genes of the cytochrome bo oxidase (cyoABCD) cluster delayed post-embryonic development and activated mitochondrial stress responses in *C. elegans* (Govindan et al., 2015). Cytochromes function in the aerobic respiratory chain, although these mutants are still capable of aerobic respiration. The authors propose that excessive ROS production in these mutants activates the $UPR^{mt}$ in *C. elegans,* resulting in delayed development (Govindan *et al.*, 2015). This presents another potential mechanism whereby respiration-deficient bacteria may alter ageing in the host, given the connection between the activity of mitochondrial homeostatic pathways and lifespan. However, the relevance of ROS production related to respiratory metabolism for the effects of *B. s.* Δ*atp* on *C. elegans* is questionable; the $UPR^{mt}$ was not activated by this diet in young adult *C. elegans* (see below), and deletion of ATP synthase in *B. subtilis* need not be accompanied by delayed development (as shown in Chapter 6). It is, however, possible that differential ROS production is relevant in this system potentially via a different mechanism.

### 3.5.4 FUdR altered the metabolism of *B. subtilis* and the lifespan of *C. elegans*

The inclusion of FUdR altered the pattern of lifespan induced by the different diets dramatically. Firstly, lifespan was considerably extended by the presence of FUdR in animals fed on all diets. This was more apparent in Fig. 3. 6A, where bacteria were not concentrated -Glu, and where nematodes were exposed to glucose from L1 until death. The mean lifespan of *C. elegans* fed on *B. s.* MS(-Glu) and Δ*atp*(-Glu) was extended by 15% and 32%, respectively, by addition of FUdR. Secondly, a diet of *B. s.* Δ*atp* was beneficial both -Glu and +Glu with FUdR present. This was true for both variations of the lifespan assays with FUdR, whereas lifespan was not extended by *B. s.* Δ*atp* when FUdR and glucose were absent. Finally, the results of Fig. 3. 6 indicate that exposure to glucose increased lifespan, but only when it was present during larval stages L1-L4 as well as adulthood. When glucose was only present throughout adulthood (Fig. 3. 6B), it was toxic and reduced lifespan. A similar phenomenon has been observed without FUdR in *C. elegans* fed on *E. coli* and 2% glucose (Tauffenberger *et al.*, 2016); here, the toxicity of adult glucose exposure seemed to arise from the ongoing activation of mitochondrial

stress responses. Importantly, the lifespan assays in Fig. 3. 6B display an unusually sharp mid-life increase in mortality, particularly +Glu, with prolonged tails. An assumption of the log rank test is that of proportional hazards, and substantial crossing of the survival curves indicates that this is not the case. Moreover, this shape is unusual and is potentially indicative of some confounding factor in the experiment. The experiment should be repeated to clarify this. It might be possible to alter the FUdR concentration or timing of nematode exposure such that off-target effects on bacterial metabolism are minimised.

The lifespan of wild-type *C. elegans* is generally considered to be unaffected by FUdR. One study (Lezzerini *et al.*, 2015) reported lifespan experiments of *C. elegans* fed on *B. subtilis* in the presence of FUdR, with no apparent irregularities. Moreover, in another study the effect of FUdR addition on the growth and biofilm proficiency of a range of different *B. subtilis* strains was compared (Donato *et al.*, 2017). The authors found no indication of drug-bacterial strain interaction, nor was there an influence of FUdR addition on *C. elegans* lifespan. However, changing the bacterial diet of *C. elegans* can potentially have a key effect on nematode physiology in the presence of FUdR. In one study (García-González *et al.*, 2017), different bacterial species exerted different effects on the response of *C. elegans* to chemotherapeutics including FUdR. Changes in bacterial ribonucleotide metabolism were identified as central to the differential effects of different bacterial species on the efficacy of this chemical. Although the main focus has been on progeny production, it is conceivable that different bacterial strains or mutants could influence lifespan in *C. elegans* differently to the wild-type strain, simply as a result of differences in the processing of FUdR. Moreover, high glucose diets have increased osmolarity (Gusarov *et al.*, 2017), and FUdR activates stress response pathways in *C. elegans,* to confer hormetic resistance to osmotic stress and lifespan extension (Anderson *et al.*, 2016). This possibly explains why glucose extended lifespan even in MS-fed individuals, in Fig. 3. 6A. For these reasons, the use of FUdR for a particular system should be validated by demonstrating that the effects of interest are present even in the absence of this chemical. In this case of this work, this could not be demonstrated, and so FUdR was excluded from further analysis.

Without FUdR, a hard extracellular matrix formed over the surface of areas of the bacterial lawns +Glu. The influence of these factors on the longevity of *C. elegans* fed on the mutant diet +Glu will be further explored in Chapter 4.

### 3.5.5 *B. s.* Δ*atp*(+Glu) improved the health of *C. elegans*

Measuring the health of *C. elegans* as it ages is crucial, especially since the link between longevity and health has been questioned by a number of studies (Tissenbaum, 2012). Along with their extended lifespan, the frequency of thrashing was higher in *C. elegans* that were fed on the ATP synthase mutant bacteria. This confirmed that *B. s.* Δ*atp*(+Glu)-fed individuals are healthier for longer in comparison to those fed *B. s.* MS, as well as being longer-lived. It is not clear at this point for exactly how long this health improvement is sustained, or whether the longevity of these individuals entails an extension of late-life frailty as has been reported for several long-lived strains of *C. elegans*. More should be done to characterise the muscular health of these individuals, perhaps by extending the assay into later life or by using a fluorescent reporter strain of *C. elegans* to directly observe muscular integrity with age.

In a recent study in which motility was categorised into different classes, early-life movement (Class A) did not effectively predict late-life movement (Newell Stamper *et al.*, 2018). This indicates that it is not an effective marker for healthspan. However, the assay employed in this work encompassed what this group defined as mid-life (Class B) movement, characterised by a lack of spontaneous motility. By day 10, spontaneous movement was minimal in animals fed on each of the *B. subtilis* diets, yet thrashing was still increased in mutant-fed nematodes. The assay therefore likely gives a good indication of the health of the animals as they age. Interestingly, 2% glucose has previously been shown to reduce thrashing dramatically in *E. coli*-fed *C. elegans,* and lead to a rapid crash in motility prior to day 5 of adulthood (Seo *et al.*, 2018). However, as described above, glucose did not affect, and if anything increased, the motility of *C. elegans* fed on *B. s.* MS into mid-adulthood. This demonstrates that the lack of glucose toxicity extends to measures of health as well as lifespan in animals fed on these *B. subtilis* strains.

### 3.5.6 Stress responses were not induced by *B. subtilis* Δ*atp*(+Glu)

As well as dietary restriction, a possible explanation for certain phenotypes described in this chapter is an alteration of stress responses in *C. elegans.* Nematodes fed on *B. s.* Δ*atp*(+Glu) have certain phenotypes including longer lifespan (and smaller body size – explored in Chapter 4), associated with stress-induced hormesis. Mitochondrial stress has also been associated with delayed post-embryonic development (Govindan *et al.*, 2015). Interestingly, mild mitochondrial

stress seems to be associated with smaller adult body size (Rea *et al.*, 2007) and the expression of stress response genes in *C. elegans*.

These pathways also influence lifespan and ageing in *C. elegans.* One study reported that increasing *hsp-6* copy number extends the mean lifespan of *C. elegans* by around 40% compared to the wild-type (Yokoyama *et al.*, 2002). Knockdown of *hsp-6* leads to reduced lifespan, reduced motility and symptoms of premature ageing including mitochondrial fragmentation (Kimura *et al.*, 2007). Moreover, induction of *hsp-6* and *gst-4* is also associated with pharmacological or genetic inhibition of the mitochondrial respiratory chain (MRC) in *C. elegans*, which promotes longevity. On this basis, the expression of *hsp-6* and *gst-4,* as well as size measurement, have been used as markers for anti-aging interventions working through mitochondrial stress (Maglioni *et al.*, 2015). However, other reports have linked the activation of these pathways to shortened lifespan. Indeed, glucose exposure upregulates the expression of *Phsp-6::gfp* (Tauffenberger *et al.*, 2016) and *Pgst-4::gfp* (Zhu et al., 2016) in *C. elegans* fed on *E. coli.* Interestingly, exposure of *C. elegans* larvae fed on *E. coli* to 2% glucose increased their lifespan in one study (Tauffenberger *et al.*, 2016), seemingly by hormetic activation of the $UPR^{mt}$, as judged by elevated expression of *Phsp-6::gfp*. However, continued $UPR^{mt}$ activation in adults (day 1 onwards) abolished this longevity. As such, long-lived animals might be expected to have low adult induction of the $UPR^{mt}$.

The induction of *gst-4* and *hsp-6* was measured to investigate the activation of stress responses in *C. elegans.* Dietary glucose upregulated the expression of *Phsp-6::gfp* at day one of adulthood in nematodes fed *E. coli*. However, glucose did not upregulate *Phsp-6::gfp* when *C. elegans* was fed on *B. subtilis,* and expression was particularly low in animals fed on *B. s.* Δ*atp*(+Glu). The absence of *Phsp-6::gfp* upregulation in *C. elegans* fed on *B. subtilis* MS(+Glu) mirrors the failure of glucose to reduce the lifespan of *C. elegans* fed on this diet. The presence of glucose similarly induced the expression of *Pgst-4::gfp* when *C. elegans* was fed on *E. coli* OP50. Once again, reporter expression was low in *C. elegans* fed on *B. s.* Δ*atp*(+Glu). However, expression was dramatically increased in *C. elegans* fed on *B. s.* MS(+Glu). This indicates that these animals were undergoing a greater level of stress, which was alleviated by feeding on the ATP synthase mutant. One explanation for this is that animals fed on Δ*atp*(+Glu) were being exposed to less glucose as a consequence of greater glucose removal from the NGM, in line with the enhanced level of glucose uptake by ATP synthase-deficient *B. subtilis*

(Santana *et al.*, 1994). Therefore, how these diets influence the direct sensing of glucose in *C. elegans,* and the effect of this on lifespan, is an important area that remains to be investigated. Alternatively, Δ*atp*(+Glu) is preventing upstream mechanisms which lead to glucose-induced oxidative stress and activation of stress responses. Importantly, since their lifespan is beyond that of nematodes without glucose, attenuation of glucose toxicity alone cannot account for the longevity of animals fed on Δ*atp*(+Glu), and other pro-longevity mechanisms must be involved.

Expression of *Pgst-4::gfp* in animals fed on *B. s.* MS(+Glu) was also strikingly high compared to those fed *E. coli* OP50. It could be speculated that this lowers the level of ROS and therefore renders glucose completely unable to reduce lifespan in *B. subtilis*-fed animals. However, as described, the role of ROS in the toxicity of glucose is controversial. Previously, elevated ROS was only observed in old glucose-exposed animals (day 15); other studies argue that glucose exposure is actually associated with lowered ROS (Gusarov *et al.*, 2017; Schulz *et al.*, 2007). Recently, a clear link has been made between elevation of oxidants and a reduction in the toxicity of glucose (Gusarov *et al.*, 2017). The authors find that glycogen stored following high glucose exposure acts as a potent antioxidant by elevating glutathione reduction. A low level of exogenous oxidants, including paraquat and diamide, was able to rescue the reduced lifespan of glucose-exposed *C. elegans* by depleting glycogen stores. Since *Pgst-4::gfp* upregulation is likely to reflect heightened ROS production in animals fed on *B. s.* MS(+Glu), a similar process of glycogen depletion by high ROS production might be responsible for preventing glucose-mediated lifespan reduction.

Overall, the screen indicates that feeding on *B. s.* ∆*atp*(+Glu) is associated with a reduced activity of oxidative and mitochondrial stress responses. There was also a statistically significant downregulation of *Phsp-6::gfp* expression in nematodes fed on ∆*atp*(-Glu) compared to MS(-Glu), though this was less dramatic than what was observed +Glu. The hypothesis that *B. s.* ∆*atp* induces a hormetic lifespan extension through adult induction of stress responses is not supported by these results. Instead, limited activity of these stress responses potentially plays an important role in longevity. It is also possible that the induction of other stress responses is responsible. It should be noted that one study (Bennett *et al.*, 2014) demonstrated that the induction of *Phsp-6::gfp* was not predictive of longevity for a range of RNAi clones. Extending the screen to include other reporter genes,

perhaps those of the heat shock response pathway such as *hsp-70* and *hsp-16.2*, would enable further testing of this hypothesis.

### 3.5.7 Stress responses and dietary restriction

In *C. elegans,* the relationship between DR and stress responses is complex. DR-induced longevity has been suggested to result from hormetic upregulation of oxidative stress response pathways (Zhou *et al.*, 2011), through SKN-1 amongst others (Bishop and Guarente, 2007). DR also stimulates the heat shock response through *hsp-70* regulation (Raynes *et al.*, 2012). However, although the $UPR^{mt}$ is activated by axenic medium, neither this nor ROS-mediated mitochondrial hormesis is required for axenic culture to mediate DR-induced longevity (Cai *et al.*, 2017). Similarly, SOD and catalase are more active in *eat-2* mutants, but SOD knockouts in diet-restricted nematodes did not affect lifespan (Zhou *et al.*, 2011). Therefore, while DR is often associated with upregulated stress responses, which pathways are involved and whether they are causal for longevity is unclear.

These data do, however, provide evidence that animals fed on *B. s.* Δ*atp*(+Glu) are not undergoing starvation. The expression of *Pgst-4::gfp* is upregulated by nutrient deprivation in *C. elegans* (Tao *et al.*, 2017). Antioxidant responses are mounted as a defence against starvation-induced ROS. Interestingly, *gst-4* is a direct target of SKN-1, and the *gst-4* reporter construct is often used as an indirect readout for SKN-1 transcriptional activity (Detienne *et al.*, 2016). Here, the expression of this reporter was low in animals fed on *B. s.* Δ*atp* with or without glucose. This shows that SKN-1-mediated DR responses are unlikely to be involved in the longevity or health of *C. elegans* fed on the mutant diet. This also provides further context for the expression of *Pacdh-1::gfp;* although this reporter can be reduced through starvation in *C. elegans,* lack of *gst-4* upregulation argues against a starvation response. The possible induction of milder lifespan-extending DR responses in these animals is explored in Chapter 4.

### 3.5.8 Evaluating the use of concentrated bacterial lawns

Concentrating the bacteria was conceived as a way of ensuring that nematodes fed on *B. s.* Δ*atp*(-Glu) were not experiencing food shortages. It was surprising to observe that concentrating the bacteria -Glu induced mitochondrial

stress (higher expression of *Phsp-6::gfp*) in day 1 adults. This highlights how changing the physico-chemical properties of the bacterial lawn can affect the stress response of *C. elegans,* and also raises the concern that concentrating the bacteria might have introduced a confounding variable into the experiments. The evidence currently indicates, however, that concentrated and unconcentrated bacteria affected *C. elegans* in similar ways. *C. elegans* were fed on *B. s.* MS(-Glu) that was concentrated throughout their whole life (Appendix Fig. A1) or only during development (Fig. 3. 4), or alternatively was not concentrated at all (Fig. 3. 3B). In each case, lifespan was similar (18-19 days mean, 18-20 days median).

The lifespan of *C. elegans* fed on Δ*atp*(-Glu) was also similar regardless of whether concentrated or unconcentrated bacteria were present. However, when the effect of unconcentrated bacteria on lifespan was investigated, concentrated bacteria were provided throughout larval development (Fig. 3. 4). The potentially confounding effect of concentrating Δ*atp*(-Glu) lawns is discussed further in section 4.5, Chapter 4, in which evidence is presented that dietary restriction responses are activated in *C. elegans* fed on unconcentrated Δ*atp*(-Glu).

As noted, vulval explosion was more prevalent in the absence of glucose than in its presence during the lifespan experiment presented in Fig. 3. 3B, and became less prevalent in animals fed on *B. s.* MS(-Glu) compared to Δ*atp*(-Glu) when both lawns were concentrated (Appendix Fig. A1). The frequency of explosion in these animals (~16%) was similar to the frequency usually observed in *C. elegans* fed *E. coli* at 20 °C (>20%) (Leiser *et al.*, 2016). A recent study has shed light on the underlying causes of this phenotype, which is usually considered an 'unnatural' cause of death (induced by environmental or genetic factors) and is therefore censored in lifespan assays (Leiser *et al.*, 2016). The authors suggest that age-related vulval integrity defects (or Avid) are in fact an ageing-related marker of reduced lifespan and healthspan, and that the censoring of this phenotype might confound lifespan analyses. Recording Avid individuals as deaths rather than censoring them would have shifted the curves to the left (reducing the recorded lifespan of animals fed without glucose in this work). Moreover, the low frequency of Avid +Glu indicates that *C. elegans* was healthier when fed on both *B. subtilis* diets in the presence of glucose. The thrashing data, in contrast, suggest that animals fed on Δ*atp* without glucose were the healthiest of all individuals (discussed below). This contradiction emphasises the need for multiple healthspan measures. Of

potential importance is that Δ*atp*(-Glu) was concentrated during the lifespan assays in which Avid was relatively frequent but was unconcentrated during the thrashing assays. Currently, no data is available regarding the frequency of Avid as distinct from other causes of censoring in nematodes fed on lawns of Δ*atp*(-Glu) that were unconcentrated. Ultimately, in order for Avid to be an effective healthspan marker, a better understanding of its relationship to natural ageing and more rigorous scoring of this phenotype into different severity classes will be necessary.

### 3.5.9 *B. s.* Δ*atp*(-Glu) improved *C. elegans* health without increasing lifespan

The work in this chapter focuses on understanding the effect of *B. s.* Δ*atp* on *C. elegans* specifically in the presence of glucose. However, there were clear indications that *B. subtilis* Δ*atp* altered the physiology of *C. elegans* even -Glu. Although lifespan was unaffected by the mutant -Glu, other phenotypes – including development, expression of *Pacdh-1::gfp* and motility – were strikingly altered. Whilst *C. elegans* fed on Δ*atp*(+Glu) were more motile in very early adulthood (day 1), Δ*atp*(-Glu)-fed individuals had overtaken them by day 4 and experienced very little decline in thrashing rate throughout the period of observation. When compared to animals fed on *B. s.* MS(-Glu), the positive effect of Δ*atp*(-Glu) was even more dramatic. No lifespan extension was observed in nematodes fed on *B. s.* Δ*atp*(-Glu), even when the lawns were unconcentrated as they were throughout the thrashing assay (Fig. 3. 4) (although animals were fed on concentrated lawns during larval development in the lifespan assay).

The experiments presented here therefore offer an interesting example of improved health without an increase in lifespan. The lifespan of *C. elegans* fed on *B. s.* MS(-Glu) and *B. s.* Δ*atp*(-Glu) was the same, but the health of the latter group was markedly improved. Whether longevity entails improved healthspan has been a matter of some debate, and in particular whether longer lifespan translates into greater health in old age. Key to this argument is the distinction between an improvement in *chronological* healthspan in age-matched individuals, compared to a *proportionate* increase in healthspan. Some studies have provided support for the idea that lifespan positively correlates with health, whereas others indicate that longevity leads to a disproportionately longer period of frailty and so can be uncoupled from health (Newell Stamper *et al.*, 2018). For example, one study measured the relative time that different mutants of *C. elegans* – including, amongst

others, the mitochondrial mutant *clk-1, eat-2(ad1113)* and *daf-2(e1370)* – spent in a healthy versus a frail state, in order to determine the 'gerospan' of the animals. These authors assessed the health – including motility and stress resistance – of long-lived mutants late in their lifespan (Bansal *et al.*, 2015). Surprisingly, it was concluded that, although the chronological time spent in good health was improved, all of the mutants studied spent a greater proportion of their life in a frail state – an undesirable scenario.

This suggests that the processes underlying health are at least partially distinct from those regulating lifespan. However, the findings of this study were challenged by subsequent reports. Whilst certain long-lived mutants, such as the mitochondrial mutant *clk-1,* do have extended frailty, *daf-2(e1370)* animals were found to have extended healthspan as well as lifespan (Hahm *et al.*, 2015). Where these contradictions arise is not clear, but recent studies (Newell Stamper *et al.*, 2018) have employed a more comprehensive approach by classifying different types of movement, and normalizing time spent in each state to mean lifespan and not maximal lifespan (which is more variable). In fact, disproportionate frailty in long-lived *E. coli*-fed nematode mutants might simply be an artefact of bacterial pathogenicity. The extent of *E. coli* colonization is greater in wild-type *C. elegans* than in *daf-2(e1370)* individuals, which have extended late-life frailty (Podshivalova *et al.*, 2017). Preventing bacterial colonization (by feeding *C. elegans* dead *E. coli*) had the effect of extending the lifespan of wild-type *C. elegans* and increasing late-life frailty in these animals, such that there was no longer a disproportionate extension of frailty in *daf-2(e1370)* mutants. This indicates that disproportionate frailty in *daf-2(e1370)* mutants did not arise as an inevitable consequence of longevity, but occurred because wild-type animals were killed by bacterial over-proliferation before the period of frailty manifested (Podshivalova *et al.*, 2017).

There are clear indications from other studies, however, that health and lifespan can be uncoupled. One study monitored individual *C. elegans* and found that animals that appear to be in a similar condition of health can have very different lifespans, while the converse is also true (Zhang *et al.*, 2016). Examples of extended health without longevity are rare, but a recent report (Sonowal et al., 2017) showed that indole production by *E. coli* K12 has a minimal effect on lifespan, but improves healthspan – including motility, heat tress resistance and reproductive health – through the action of the aryl hydrocarbon receptor. Indoles enhanced health by regulating a set of genes that is distinct from those usually associated with

longevity, and effectively uncoupled the effect of advanced age on health by shifting the gene expression profile to a more youthful, 'healthy' pattern (Sonowal *et al.*, 2017). Ultimately, therefore, it seems that the underlying physiological causes of health decline need not overlap with those that shorten lifespan (Zhang *et al.*, 2016). The work presented in this chapter supports this notion. Because lifespan is unaffected, it seems that *B. s.* Δ*atp*(-Glu) extends proportionate as well as chronological healthspan. This is ideal, as the extended period of frailty that is often reported for long-lived mutants of *C. elegans* seems to be absent. Notably, the mortality curves of *C. elegans* fed on ATP synthase-deficient *B. subtilis* have the typical sigmoidal shape and do not have any unusual features which might reflect, for example, a sudden increase in mortality following a period of enhanced health. The survival distribution was therefore largely unaffected by the fact that most individuals were in a better state of health.

While the mechanisms underlying increased lifespan in *C. elegans* have been well characterised, those that influence healthspan are largely unknown. It will be valuable to expand the range of assays used for quantifying health, and to encompass resistance to stress as in some previous studies (Bansal *et al.*, 2015) (the topic of uncoupling lifespan and health is further explored in Chapter 6).

### 3.5.10 What is the mechanism(s) for lifespan extension by *B. s.* Δ*atp*(+Glu)?

As described, *B. s.* Δ*atp* improved the health of *C. elegans* even without glucose. It can therefore be concluded that the presence of glucose was not required for the beneficial effect of this diet on health. Research into the effects of *B. subtilis* lacking ATP synthase on *C. elegans* in the absence of glucose is extended and discussed further in Chapter 6.

In light of the data presented in this chapter, a number of hypotheses can be proposed to explain the longevity of animals fed on *B. s.* Δ*atp*(+Glu):

1. *B. s.* MS(+Glu) produces metabolites that shorten the lifespan of *C. elegans.*
2. *B. s.* Δ*atp* produces pro-longevity metabolites in the presence of glucose.
3. *B. s.* Δ*atp*(+Glu) upregulates glucose protection mechanisms or otherwise enables glucose to directly extend *C. elegans* lifespan.
4. Growth of *B.* s. Δ*atp*(+Glu) obstructs feeding and induces DR to extend lifespan.

**Is the master strain harmful to *C. elegans*?** The evidence for extended lifespan and/or enhanced health when *C. elegans* was fed on a diet of *B. s.* Δ*atp* could be attributed to some probiotic effect of this diet. Alternatively, it is possible that the MS diet is detrimental to *C. elegans,* and negatively affects lifespan and certain aspects of nematode physiology. Since *B. s.* Δ*atp*(+Glu) is deficient in respiratory metabolism, it seems plausible that it is lacking in an interaction which usually reduces lifespan. One possibility is that the mutant does not produce certain metabolites which usually shorten the lifespan of *C. elegans* in the presence of glucose. Not only would this imply, however, that the effect of glucose on the lifespan of *C. elegans* is positive, which is inconsistent with previous reports (although still a possibility), other studies have shown that *B. subtilis* is a non-pathogenic, healthier diet in comparison to *E. coli* OP50 (Garsin *et al.*, 2003; Gusarov *et al.*, 2013). Importantly, *C. elegans* fed on MS(+Glu), which had a higher growth rate, had the same lifespan and motility as those fed on MS(-Glu), indicating that a negative activity of the master strain is unlikely.

**Release of pro-longevity metabolites by *B. s.* Δ*atp*(+Glu).** More likely are explanations centred on the production of beneficial metabolites by *B. s.* Δ*atp*(+Glu). These could conceivably be factors like NO and CSF, which are partly responsible for the relative health of *C. elegans* fed on *B. subtilis* relative to *E. coli* (Donato *et al.*, 2017). Alternatively, metabolites linked specifically to growth in the absence of oxidative phosphorylation might be responsible. Acetate, the production of which is doubled in ATP synthase-deficient *B. subtilis* strains (Santana *et al.*, 1994), has been reported to extend the lifespan of *C. elegans* via DAF-16 (Chuang *et al.*, 2009). Although aerobic metabolism uncoupled to ATP synthesis is prominent in respiration-deficient *B. subtilis* in glucose minimal medium, the metabolism of ATP synthase-deficient *B. subtilis* on NGM has not been defined, and fermentative pathways might be active. *B. subtilis* is capable of using oxygen and nitrate as electron acceptors, and can also grow anaerobically by fermenting sugars, without the involvement of mitochondrial electron transport. Fermentation products of *B. subtilis* include acetate, lactate, succinate, acetoin and ethanol (Ramos *et al.*, 2000), the production of which might underlie the effects of *B. s.* Δ*atp*(+Glu) on lifespan. Indeed, the release of lifespan-extending factors such as fermentation products has been suggested to elicit the positive effects of a coQ-less *E. coli* diet (Saiki *et al.*, 2008).

Aside from fermentation products, the metabolism of ATP synthase is linked to a number of pathways. This includes the production of hydrogen sulphide ($H_2S$) (Sasahara *et al.*, 1997), thiamine (Vitamin B1) (Gigliobianco *et al.*, 2013), α-ketoglutarate (α-KG) (Chin *et al.*, 2014), and pyruvate (Koch-Koerfges *et al.*, 2012; Zhu *et al.*, 2008). $H_2S$, α-KG and pyruvate can extend *C. elegans* lifespan (Chin *et al.*, 2014; Miller and Roth, 2007; Mouchiroud *et al.*, 2011), whilst *C. elegans* with thiamine transport deficiency are long-lived (de Jong *et al.*, 2004). The longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu) might therefore be mediated by shifts in these or other metabolic pathways. Samples of *B. s.* MS and *B. s.* Δ*atp* grown on NGM(-Glu) or NGM(+Glu) are currently undergoing untargeted, high-throughput metabolomic analysis by Time-Of-Flight Mass Spectrometry (TOF-MS) for the identification of pro- or anti-ageing metabolites. These candidates will be purified and added exogenously onto lawns of *B. s.* MS, and the lifespan of *C. elegans* fed on these diets will be determined.

A comparison with *E. coli* indicates that proliferation inside the *C. elegans* intestine might be important. Conceivably, glucose-stimulated growth promotes the establishment of *B. s.* Δ*atp* inside the *C. elegans* gut, where it can mediate pro-longevity effects. Fluorescent reporter strains can help to address this point (see Chapter 6).

**Potential effector mechanisms for longevity and health mediated by *B. s.* Δ*atp*(+Glu).** Possibly, the benefits of *B. s.* Δ*atp* could be mediated by shifts in stress response pathways. The low activation of stress responses seen in animals fed on Δ*atp*(+Glu) possibly plays a causal role in longevity. Alternatively, stress-induced hormesis extends the lifespan of *C. elegans* fed on *B. s.* Δ*atp*(+Glu) independently of $UPR^{mt}$ and the cytosolic oxidative stress response. In another view, the longevity of animals fed on *B. s.* Δ*atp*(+Glu) is a result of hyper-activation of glucose-protective pathways, although activation of such mechanisms has only previously been demonstrated to restore normal lifespan in the presence of glucose, rather than elicit additional longevity.

Another idea that will be explored further is that dietary restriction is responsible for the longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu). During the lifespan experiment presented in Fig. 3. 3B, *C. elegans* fed on *B. s.* Δ*atp*(+Glu) were long-lived but also had high levels of escape (24 animals were lost as opposed to only 1

animal fed on *B. s.* MS(+Glu)). A potential explanation for this is that *C. elegans* shows aversion to lawns of *B. s.* Δ*atp*(+Glu), and DR through reduced food intake is induced. *C. elegans* exhibits preferences for its bacterial food, favouring bacteria that support healthy development over pathogenic strains (Shtonda and Avery, 2006). *C. elegans* seems to prefer bacterial species with a higher growth and respiration rate (Yu *et al.*, 2015). Since the ATP synthase mutant delayed post-embryonic development and has a slower growth rate (although respiration rate is elevated by 50% in the mutant), some avoidance of *B. s.* Δ*atp* would not be surprising. Moreover, the downregulation of *acdh-1* by *B. s.* Δ*atp*(+Glu) implies a DR mechanism. These ideas are further explored in Chapter 4.

A glucose concentration of 2% was used throughout this report. However, it will be informative to experiment with varying the concentration, to ascertain whether there is a dose-dependent response or a threshold effect. Previously, concentrations ranging from 0.05% to 4% steadily reduced the lifespan of *C. elegans* fed on *E. coli* OP50 (Lee *et al.*, 2009; Seo *et al.*, 2018). It might be possible for *B. s.* Δ*atp*(+Glu) to elicit an even greater lifespan extension using a different concentration of glucose. Similarly, the effect of sugars other than glucose should be determined. Interestingly, lower concentrations of the monosaccharide fructose (1-2%) prolong *C. elegans* lifespan, whereas higher concentrations reduce lifespan and correlate with higher intestinal fat deposition (Zheng *et al.*, 2017). The lifespan of *C. elegans* fed on *B. s.* Δ*atp* in the presence of fructose and other simple sugars should be measured to determine whether the effects are specific to glucose, and to define dose-response relationships.

### 3.5.11 Conclusions and future perspectives

Currently, the benefits afforded by respiration-deficient bacteria have been attributed to a reduction in bacterial pathogenicity – either as a direct result of reduced intestinal colonisation, or through a limitation of some detrimental activity (e.g. alteration of REDOX homeostasis by reducing coQ production) (Sánchez-Blanco *et al.*, 2016). Here, however, evidence is presented that respiration-deficiency can improve health and extend lifespan in a manner that is unlikely to involve a reduction in a toxic bacterial activity and is modulated by a dietary component (glucose). Although the toxic effect of glucose on *C. elegans* is thought to be largely direct, the metabolism of bacteria in the presence of sugar can influence this, even to the extent that the addition of glucose to the growth medium

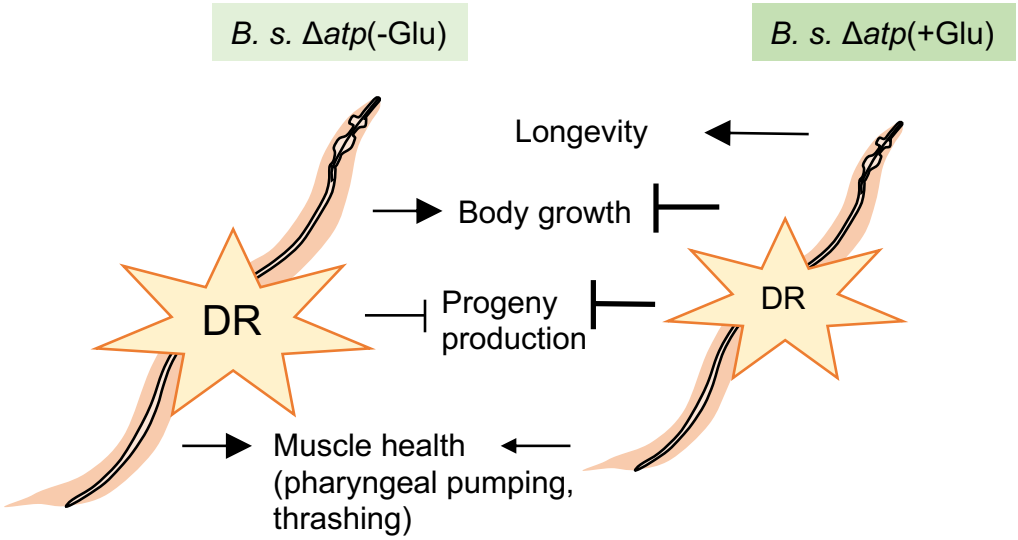
increases lifespan under certain conditions (Brokate-Llanos *et al.*, 2014). In this study, however, the mechanism entailed a reduction in the efficiency of *E. coli* infection in the presence of glucose, whereas an entirely different mechanism is likely to underlie the longevity elicited by *B. s.* Δ*atp*(+Glu).

This diet also improved health in ageing animals, fulfilling a key criterion for a probiotic intervention. Stress response pathways are also dramatically affected in the presence of glucose. It is difficult to draw conclusions regarding the causative effect of reporter expression changes on lifespan, particularly given that their induction had no consistent correlation with lifespan. For example, the expression of *Pgst-4::gfp* was much higher in *C. elegans* fed on *B. s.* MS in the presence compared to the absence of glucose, but lifespan of *C. elegans* on these diets was the same. However, the screens give an interesting indication that stress responses are being modulated in a characteristic way by feeding on *B. s.* Δ*atp*(+Glu) and will be followed up by stress resistance assays (using spore-less strains, Chapter 6).

The effects of a respiration-deficient diet on lifespan and health has not been thoroughly investigated, particularly in model systems other than *C. elegans.* However, feeding *Drosophila* on a diet of respiratory incompetent yeast (both with or without coQ production) shortened lifespan compared to the respiration-competent parent strain (Palmer and Sackton, 2003). Possibly, therefore, this phenomenon may be specific to *C. elegans.* Nevertheless, studying the effects of *B. s.* Δ*atp* on *C. elegans* provides an opportunity to understand microbiome-mediated enhancement of health and lifespan, and can potentially provide novel explanations for the beneficial effects of similar mutations in *E. coli.*

## 4 The role of dietary restriction in the longevity of *C. elegans* fed on *B. subtilis* Δ*atp*(+Glu).

### 4.1 Graphical abstract




### 4.2 Introduction

#### 4.2.1 How to elicit DR in *C. elegans*

DR has commonly been induced in *C. elegans* by altering food quantity. A number of DR paradigms centered on the periodic or constitutive restriction of bacterial availability have been developed (Heintz *et al.*, 2016). In the most extreme cases, *C. elegans* can be cultured in the absence of food (bacterial deprivation, BD) or on axenic medium (ADR). In the case of ADR, nutrients are provided through semi-defined media (Houthoofd *et al.*, 2002). The bacteria can also be serially diluted in liquid media, known as 'bacterial dietary restriction' (bDR), or liquid DR (LDR) (Kapahi *et al.*, 2017). Alternatively, the bacteria can be diluted and seeded onto the surface of solid media (sDR) (Heintz *et al.*, 2016). DR can be imposed constitutively, or it can be induced in a stage-specific manner, for example by periodically shifting the availability of food ('intermittent fasting', IF).

DR in *C. elegans* has been induced by means other than changing food quantity or composition. For example, it is possible to elicit DR by genetic

manipulation. The primary genetic mimetic of DR that is employed in *C. elegans* research is mutation of the *eat* genes. These mutants are defective in pharyngeal pumping as a result of changes in the rate, sequence or strength of muscle contraction (Lakowski and Hekimi, 1998). Of these, the most commonly targeted gene is *eat-2,* which encodes a subunit of a nicotinic acetyl-choline receptor found post-synaptically in pharyngeal muscle (Avery and Horvitz, 1989; Kapahi *et al.*, 2017). The rate of pharyngeal pumping is linearly related to food intake (Klass, 1977); *eat-2* mutation reduces the rate of pharynx pumping and leads to constitutive feeding restriction throughout the entire lifespan of the animal. Lifespan is extended in this strain by around 30% (Lakowski and Hekimi, 1998) as a result of reduced food intake and DR.

### 4.2.2 Effector mechanisms of DR in *C. elegans*

Strikingly, genes that are required for certain interventions to increase lifespan are not required for others, and to date a single unifying upstream mechanism mediating these diverse DR regimes has not been identified. Instead of a master regulator of all DR regimes, it seems that the changes in nutrient sensing that accompany food shortage work through a number of interconnected pathways to alter nematode physiology in characteristic ways (Heintz *et al.*, 2016), Fig. 4. 1.

The longevity of *C. elegans* fed on sDR requires *daf-16* (Greer *et al.*, 2007). In this DR regime, the bacteria were serially diluted and seeded onto NGM plates. In sDR conditions, AMP-activated protein kinase (AMPK) enhances DAF-16-dependent transcription, resulting in longevity and enhanced oxidative stress resistance (Greer *et al.*, 2007). In particular, the alpha-subunit of AMPK (*aak*-2) couples energy sensing and IIS pathways to lifespan (Apfeld *et al.*, 2004). Under sDR, *aak-2* is required for DR-induced longevity (Greer *et al.*, 2007). However, *daf-16* is dispensable for dietary restriction induced by *eat-2*, ADR (Houthoofd *et al.*, 2003), bDR and BD (Heintz *et al.*, 2016), and so the role of the IIS in DR is not universal.

Similarly, the role played by TOR signalling varies according to the intervention. The TOR kinase promotes growth by integrating nutrient signals, and inhibition of TOR signalling prolongs lifespan in organisms from yeast to mice (Kapahi *et al.*, 2010). The *C. elegans* TOR gene, *let-363,* is downregulated by BD dietary restriction (Thondamal *et al.*, 2014). BD activates steroid signalling through

*let-363/*TOR by increasing the expression of the cytochrome P450 enzyme DAF-9. Interestingly, reducing TOR signalling in *C. elegans* by RNAi did not further extend the lifespan of *eat-2(ad1116)* mutants, suggesting that both TOR signalling and DR induced by *eat-2* work through the same downstream pathways (Hansen *et al.*, 2007). A critical pathway that is proposed to mediate the lifespan-extending effects of mTOR inhibition and DR is macroautophagy, a process in which cellular components are targeted for lysosomal degradation. Revealingly, inhibition of autophagy-related genes including the ATG6 ortholog *bec-1* reduced the longevity of *eat-2* mutants (Kapahi *et al.*, 2017). *bec-1* expression is required for the long lifespan of *C. elegans* mutants with low TOR activity (Hansen *et al.*, 2008). Similarly, the expression of autophagy genes is increased by many of the transcription factors that are required for longevity induced by DR and mTOR, including PHA-4 (see below) and the widely expressed nuclear hormone receptor NHR-62 (Heestand *et al.*, 2013).

In order to investigate the induction of DR pathways in *C elegans,* the activity of several key genes can be monitored. SKN-1, the *C. elegans* homologue of the mammalian transcription factors NF-E2-related factors (Nrfs) regulates a number of processes in *C. elegans* through independent mechanisms, including lifespan and the response to oxidative stress (Tullet *et al.*, 2017). SKN-1 was required in the ASIs - a pair of sensory neurons located in the head - but not in the intestine where it is also expressed, in order to increase mitochondrial respiration and promote longevity of bDR animals (Bishop and Guarente, 2007). However, SKN-1 was not required for the longevity of animals maintained in liquid ADR culture (Castelein *et al.*, 2014). Similarly, both IF and sDR (Greer and Brunet, 2009) have been demonstrated to elicit longevity in *skn-1* individuals. The FOXA transcription factor PHA-4 transcription factor appears to play a role in *C. elegans* which is specific to DR, and not other ageing-associated pathways. Expression levels of *pha-4* were increased by over 80% specifically in response to DR by bacterial deprivation (Panowski *et al.*, 2007), and PHA-4 overexpression is sufficient to enhance stress resistance and increase lifespan. Adult expression of PHA-4 is required for the longevity of *eat-2* mutants, and for *C. elegans* undergoing bDR (Kapahi *et al.*, 2017; Panowski *et al.*, 2007). Finally, the expression of heat shock factor 1 (HSF-1) is required for the longevity of *daf-2(e1370)* animals, as well as for reducing proteotoxicity and extending lifespan following bDR (Steinkraus *et al.*, 2008), but is dispensable for *eat-2(ad1116)*-mediated longevity.

Longevity has been observed whether DR is initiated from hatching, from the L4 stage, during the reproductive stage or in post-reproductive (day 4) adults (Greer and Brunet, 2009; Greer *et al.*, 2007), although the timepoint at which DR is initiated can considerably alter its effects (Lee *et al.*, 2006). Similarly, DR consistently reduces fecundity when initiated prior to the end of the self-fertile period (Greer and Brunet, 2009).

In Chapter 3, data was presented which indicated that, in the absence of FUdR, a diet of *B. s.* Δ*atp* failed to extend *C. elegans* lifespan unless glucose was present in the growth media. An important question to address when an intervention improves lifespan and health of *C. elegans* is whether DR is being induced as a consequence of limited food intake. DR is a robust response induced by a broad variety of interventions which can produce many anti-ageing phenotypes in *C. elegans*. Such investigation is central to the question of whether *B. s.* Δ*atp* is a probiotic, or if its effects are conferred by a passive process of reduced food intake. In this chapter, the possibility that DR was induced in *C. elegans* fed on *B. s.* Δ*atp*(+Glu) was investigated further.

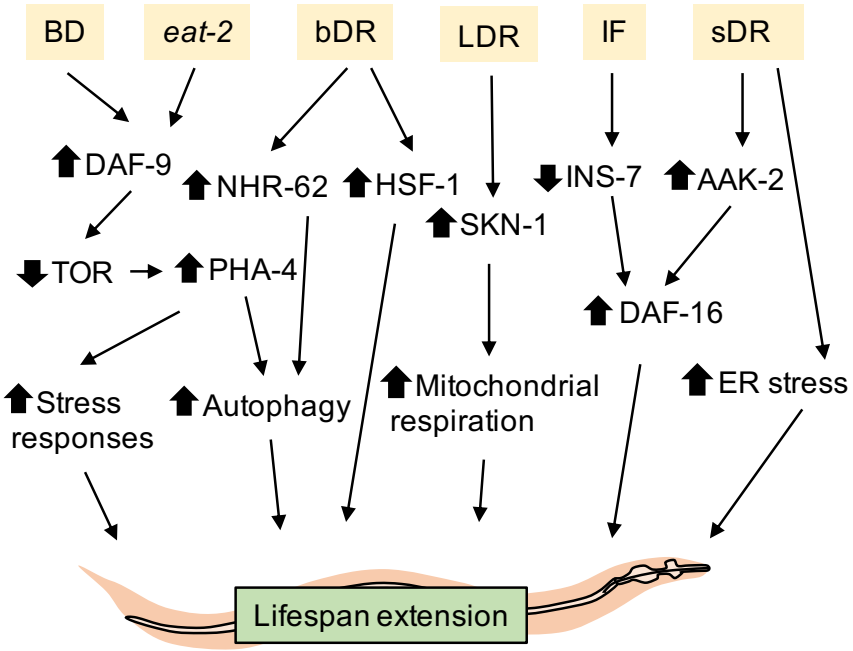



**Figure 4. 1 Signalling pathways mediating longevity and stress resistance induced by dietary restriction in *C. elegans*.** Different DR regimens are given at the top of the figure, and effector genes and processes are indicated, where known. See main text for details. Figure adapted from Kapahi *et al.* (2017).

## 4.3 Materials and Methods

### 4.3.1 Nematode growth medium (NGM)

**NGM containing chloramphenicol**

Add chloramphenicol to a final concentration of 0.7 µg/ml into liquid NGM(-Glu) or NGM(+Glu).

### 4.3.2 *B. subtilis* strains

*B. s.* MS and ∆*atp* were used throughout (Chapter 2).

### 4.3.3 *C. elegans* strains

The wild-type N2 (Bristol) strain was used unless otherwise indicated. The mutant strain DA1116 (*eat-2(ad1116*)) was also used (see Chapter 2.2).

### 4.3.4 *C. elegans* oligonucleotides

Table 4. 1 shows the primers used for *C. elegans* qRT-PCR analysis.

**Table 4. 1 Oligonucleotide primers used for *C. elegans* qRT-PCR.**

| Target | Primer | Sequence (5' – 3') | Source |
|---|---|---|---|
| *pmp-3* | Fwd | GTTCCCGTGTTCATCACTCAT | Thondamal *et al.* (2014) |
| | Rev | ACACCGTCGAGAAGCTGTAGA | |
| *let-363* | Fwd | TATAAGAAAGCACGCGGCAC | |
| | Rev | CAGATACGGTATTAGTTCGCC | |
| *daf-9* | Fwd | GGGCAAATATCGTGTTATGG | |
| | Rev | GATTGGATCTCCTCTGTTCA | |
| *act-1* | Fwd | ACGACGAGTCCGGCCCATCC | Zhang *et al.* (2012) |
| | Rev | GAAAGCTGGTGGTGACGATGGTT | |
| *daf-16* | Fwd | TCCTCATTCACTCCCGATTC | J. Lee *et al.* (2015) |
| | Rev | CCGGTGTATTCATGAACGTG | |
| *sod-3* | Fwd | GGATGGTGGAGAACCTTCAA | |
| | Rev | AAGGATCCTGGTTTGCACAG | |
| *aak-2* | Fwd | TGCTTCACCATATGCTCTGC | |
| | Rev | GTGGATCATCTCCCAGCAAT | |
| *skn-1* | Fwd | TCAGGACGTCAACAGCAGAC | |
| | Rev | CGTGGAGATTCCGAAGAGAG | |
| *hsf-1* | Fwd | AGCAGCACGTCGTTATGTTC | |
| | Rev | TACTGGAAGCTTGTCGTCGT | |
| *daf-2* | Fwd | GCCCGAATGTTGTGAAAACT | |
| | Rev | CCAGTGCTTCTGAATCGTCA | |
| *bec-1* | Fwd | ACGAGCTTCATTCGCTGGAA | Wilhelm *et al.* (2017) |
| | Rev | TTCGTGATGTTGTACGCCGA | |
| *pha-4* | Fwd | ATCTTGGCCTAATTGACCCATC | Higashibata *et al.* (2006) |
| | Rev | TCATCTGTGCTTGCGTGGTT | |
| *mtl-1* | Fwd | GGCTTGCAAGTGTGACTGCA | Smolentseva *et al.* (2017) |
| | Rev | CGCAGCAGTTCCCTGGTGT | |
| *hsp-70* | Fwd | AATGAACCAACTGCTGCTGCTCTT | |
| | Rev | TGTCCTTTCCGGTCTTCCTTTTG | |

### 4.3.5 Pouring NGM(-Glu) and NGM(+Glu) for *C. elegans* qRT-PCR

For the culturing of *C. elegans* for qRT-PCR samples, 10 cm NGM(-Glu) and NGM(+Glu) plates were poured, containing 30 ml solid NGM. These were left to dry at 20 °C for two weeks prior to use. Throughout the rest of the chapter, 6 cm NGM plates were used as previously described.

### 4.3.6 Growth of bacteria on NGM

#### 4.3.6.1 Seeding NGM

NGM(-Glu) and NGM(+Glu) were seeded as described in Chapter 3. As indicated, bacteria were occasionally concentrated (conc.) in the absence of glucose also as described in Chapter 3.2.

Larger (10 cm) NGM(-Glu) plates for *C. elegans* qRT-PCR samples were seeded with 600 μl of fresh *B. s.* MS and Δ*atp* overnight culture. This volume of *B. s.* Δ*atp* was seeded twice. 10 cm NGM(+Glu) plates were seeded with 300 μl of both strains, after preparing a 2:3 dilution in $_{dd}H_20$ as usual. Plates were left to grow overnight at 20 °C before the introduction of nematodes.

#### 4.3.6.2 Seeding NGM containing 0.7 μg/ml chloramphenicol

NGM(-Glu) and (+Glu) were seeded with 100 μl of *B. s.* MS or Δ*atp* from a fresh overnight culture. *B. s.* Δ*atp* was seeded three times with this volume onto NGM(-Glu) plates to ensure sufficient food for the nematodes (300 μl in total). Plates were left at 20 °C for 48 hrs to grow before the introduction of nematodes.

### 4.3.7 Imaging of *B. subtilis* cells and size measurements

NGM(-Glu) and (+Glu) plates were seeded with the relevant *B. subtilis* strain and were left to grow for two days at 20°C. Samples were removed with a platinum pick from lawns of each bacterial strain. Samples were added into a small volume of M9 and re-suspended by vortexing, and 10 μl was pipetted onto an agar pad, formed from 2% agarose on a glass slide. A coverslip was placed on the sample, and DIC images were taken with a 100x oil immersion objective. Cell measurements were taken in Image J v 2.0.0 using the line tool for 103 cells (width) or 183 cells (length) per condition.

### 4.3.8 Nematode experimentation

Table 4. 2. gives a summary of the hypotheses that were addressed and the conclusions that were reached in this chapter. 20-25 animals were cultured per NGM plates unless otherwise stated.

#### 4.3.8.1 Lifespan assays

Lifespan assays were performed essentially as described in Chapter 3. Where chloramphenicol was added into the NGM, nematodes were raised in the absence of antibiotic and glucose until the early L4 stage (48 hrs later). Nematodes were then introduced onto NGM(+/-Glu) plates seeded with 0.7 μg/ml chloramphenicol.

#### 4.3.8.2 Pumping rate measurements

The rate of pharynx pumping ($min^{-1}$) was calculated as the number of pharyngeal terminal bulb contractions in one minute. Pumping was quantified at the indicated adult timepoints. At least 10 age-matched individuals were examined for each time point, across two independent trials. A Carl Zeiss Axio Zoom.V16 microscope was used for all observations.

#### 4.3.8.3 Nematode size measurements

The body size of *C. elegans* fed on *B. s* MS or Δ*atp* was measured as described in Moore *et al.* (2013). Animals were synchronised and introduced onto each diet. Bacteria were concentrated -Glu. Images of the nematodes fed on *B. s.* MS and Δ*atp* on NGM+/- plates were taken at days 1, 3, 5, 7 and 9 of adulthood. Nematodes were picked onto an empty NGM plate, away from bacteria, and aligned prior to imaging. The volume of individual nematodes was determined using the WormSizer plugin for ImageJ software (ver.1.1.2) (Moore *et al.*, 2013), and the recognition of each nematode was passed or failed by eye. For each condition, 57-93 nematodes were analysed.

#### 4.3.8.4 Measuring viable progeny production

*C. elegans* were synchronised and introduced as L1s onto *B. s.* MS or Δ*atp.* Bacteria were concentrated -Glu. One nematode was kept on each NGM plate following transfer of L4s onto NGM(-Glu) or NGM(+Glu) plates to enable the number of progeny per animal to be counted. Nematodes were transferred onto fresh plates every day until the end of the reproductive period. Progeny were left to develop for 48hrs before counting. Hermaphrodites that bagged or escaped were censored from the analysis. Brood sizes were calculated by combining the daily egg lay of 15-18 individuals fed on each of the diets. The total brood size of animals fed on *B. s.* Δ*atp*(+Glu) could not be assessed due to escape prior to cessation of egg laying.

#### 4.3.8.5 Collection of samples for qRT-PCR

*C. elegans* were synchronised and introduced as L1s onto 10 cm NGM(-Glu) plates. 800 L1s were introduced onto each plate. At the L4 stage, 400 animals were transferred onto 10 cm NGM(-/+Glu) plates seeded with the respective diet (120-150 animals per plate). Two days later, 100-120 day 2 adults from each of the four diets were picked into 200 μl sterile M9 buffer, washed from progeny and eggs with 300 μl M9 2-3x by letting adults sink and removing the supernatant, and suspended in 200 μl TRIzol reagent (Thermo Fischer Scientific). This was repeated so that 300-400 nematodes were collected for each diet. Animals were immediately flash frozen in liquid nitrogen and stored at – 80 °C until RNA extraction. Samples were collected for two independent biological replicates.

#### 4.3.8.6 Extraction of RNA from *C. elegans*

Nematode samples were freeze cracked with dry ice three times, with vortexing during defrosting. 120 μl chloroform was added to each 600 μl sample, followed by vortexing for 15 seconds and incubation at room temperature for 5 minutes. Samples were then centrifuged at 12,000 g for 15 mins at 4 °C, and the upper aqueous phase was transferred to a new tube. 300 μl of 100% isopropanol was added to each sample, and the samples were mixed and incubated at room temperature for 10 minutes. Samples were centrifuged at 12,000 g for 10 mins at 4 °C, and the supernatant was removed. Pellets were washed with 400 μl of 70% ethanol, and centrifuged at 10,000 g for 5 mins at 4 °C. The ethanol was removed, and the pellets were air dried for 10 mins and dissolved in 30 μl nuclease-free $H_2O$. Samples were stored at -80 °C until DNase treatment, cDNA synthesis and qRT-PCR. This proceeded as described in Chapter 2.2.

### 4.3.9 Microscopy

Lifespan, fecundity and lawn location observations were made using an Olympus SZ61 stereo microscope. Cell images were taken with a Carl Zeiss Axio Observer Z1 epifluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). All other images were taken using a Carl Zeiss Axio Zoom.V16 Fluorescence zoom microscope and Zen software.

### 4.3.10 Statistical analysis

Analysis of lifespan data was as described in Chapter 3.2. The location of *C. elegans* in different zones of the NGM plate on each diet was compared using Pearson's Chi-square test. Cell size was compared using the Kruskal-Wallis test followed by Dunn's *post-hoc* test (false discovery rate multiple correction). The body volume data were analysed with a non-parametric Kruskal-Wallis test followed by Dunn's *post-hoc* test with false discovery rate multiple correction. Data for daily progeny production were compared using one-way ANOVA (day 1 and 3) or the non-parametric Kruskal-Wallis test (days 2 and 4), followed by Tukey's HSD or Dunn's *post-hoc* tests, respectively. Pharynx pumping data were subjected to a non-parametric Kruskal-Wallis test followed by Dunn's *post-hoc* test (FDR *p*-value correction). All statistical analysis was conducted using R version 3.1.1.

**Table 4. 2 Summary of experimental conditions for the experiments in Chapter 4.** unconc, unconcentrated; conc, concentrated. Where *B. s.* Δ*atp* was not concentrated, 150 µl were seeded onto NGM(-Glu) (scaled up for qRT-PCR, see above) to provide sufficient food. 50 µl of all other bacterial strains were seeded onto NGM(-Glu) or (+Glu) plates (whether concentrated or unconcentrated). DR, dietary restriction.

| Figure | Hypothesis / question | Bacterial strains | *C. elegans* culture conditions | | Growth* | Conclusion |
|---|---|---|---|---|---|---|
| | | | L1-L4 | Adulthood | | |
| **4.2** | *B. s.* Δ*atp*(+Glu) induces DR;<br>Is body size affected by Δ*atp*(+Glu)? | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: conc. | -Glu: conc.<br>+Glu | 24 hrs | Body size was reduced by *B. s.* Δ*atp*(+Glu), consistent with DR. |
| **4.3** | *B. s.* Δ*atp*(+Glu) induces DR;<br>Is fecundity affected by Δ*atp*(+Glu)? | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: conc. | -Glu: conc.<br>+Glu | 24 hrs | Egg laying was delayed by *B. s.* Δ*atp*(+Glu), consistent with DR. |
| **4.5** | *B. s.* ∆*atp* cells are inedible | *B. s.* MS<br>*B. s.* Δ*atp* | - | - | 48 hrs | Cell size does not obstruct feeding. |
| **4.6** | *B. s.* Δ*atp*(+Glu) induces DR;<br>*C. elegans* consume less of *B. s.* Δ*atp*(+Glu) | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: unconc. | -Glu: unconc.<br>+Glu | 24 hrs | Pharyngeal pumping rate was unaffected by *B. s.* Δ*atp*(+Glu), indicating similar levels of food intake. |
| **4.9** | *C. elegans* avoids *B. s.* Δ*atp*(+Glu) | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: conc. | -Glu: unconc.<br>+Glu | 24 hrs | *C. elegans* avoided areas of lawn overgrowth. |
| **4.10** | Overgrowth of the *B. s.* Δ*atp*(+Glu) lawn elicits longevity | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: conc. | -Glu: unconc.<br>+CHLOR.<br>+Glu: +CHLOR.** | 48 hrs | Abundant growth of *B. s.* Δ*atp*(+Glu) was required for longevity. |

| Figure | Hypothesis / question | Bacterial strains | *C. elegans* culture conditions | | Growth* | Conclusion |
|---|---|---|---|---|---|---|
| | | | L1-L4 | Adulthood | | |
| **4.11** | *B. s.* Δ*atp*(+Glu) induces DR; *B. s.* Δ*atp*(+Glu)-induced longevity and *eat-2* mutation are not additive | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: conc. | +Glu | 24 hrs | These factors were additive, indicating separate mechanisms for longevity. |
| **4.12** | DR pathways are activated by *B. s.* ∆*atp*(+Glu) | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: unconc. | -Glu: unconc.<br>+Glu | 24 hrs | *B. s.* ∆*atp*(-Glu) activated DR-related pathways in *C. elegans;* this was lessened by the addition of glucose. |
| **4.13** | A diet of *B. s.* ∆*atp*(+Glu) is associated with upregulation of biofilm-responsive genes | *B. s.* MS<br>*B. s.* Δ*atp* | -Glu: unconc. | -Glu: unconc.<br>+Glu | 24 hrs | *B. s.* ∆*atp*(+Glu) did not upregulate genes implicated in the probiotic effect of biofilm formation. |

* the length of time in which bacteria were left to grow on NGM prior to the introduction of nematodes at 20 °C.

** 0.7 μg/ml chloramphenicol in the NGM.

## 4.4 Results

### 4.4.2 Nematodes fed on *B. s.* Δ*atp*(+Glu) displayed phenotypes associated with dietary restriction

DR can elicit characteristic morphological and physiological effects in *C. elegans*. These commonly include a reduction in body size and reduced fecundity (Chen *et al.*, 2009). Therefore, these phenotypes were investigated in animals fed on the *B subtilis* diets.

#### 4.4.2.1 A diet of *B. s.* Δ*atp*(+Glu) reduced *C. elegans* body size

Differences in body volume (picoliters, pL) of nematodes fed on each diet were quantified (Fig. 4. 2). Bacteria were concentrated in the absence of glucose. Nematode diet significantly affected body volume at each timepoint, as determined by a Kruskal-Wallis test ($p < 0.0001$). The output of statistical analysis including test statistics and *p* values are given in Appendix Table B1. Following omnibus tests, Dunn's *post-hoc* was used for multiple comparisons.

On day 1 of adulthood, the average body size of animals fed on *B. s.* MS(+Glu) (N = 57) was around 25% less than that of animals fed on MS(-Glu) (N = 73) ($p < 0.0001$). Similarly, animals fed on Δ*atp*(+Glu) were around 25% smaller (N = 48) than those fed on Δ*atp*(-Glu) (N = 93) ($p < 0.0001$). Whether nematodes were fed on *B. s.* MS or Δ*atp* did not affect body size, in the absence or presence of glucose ($p = 0.078$, $p = 0.031$, respectively). By day 3, however, a reduction in body size (~35% compared to animals fed on *B. s.* MS) in *B. s.* Δ*atp*(+Glu)-fed individuals had become apparent ($p < 0.0001$). This reduction in size was maintained throughout the experiment until day 9 of adulthood. From day 3, individuals fed on MS(+Glu) were 21-35% larger than those fed *B. s.* Δ*atp*(+Glu) ($p < 0.0001$) but were still smaller than those fed on ∆*atp*(-Glu) (except on day 3 of adulthood, $p = 0.52$). The body size of *B. s.* Δ*atp*(-Glu)-fed individuals was not reduced. In fact, individuals fed without glucose maintained a larger body volume throughout the period of observation, with those fed on *B. s.* Δ*atp*(-Glu) consistently larger (12-18%) than those fed MS(-Glu) ($p < 0.0001$) (except for day 5, $p = 0.86$). Overall, the reduction in body size observed only in animals fed *B. s.* ∆*atp*(+Glu) is consistent with the hypothesis that these animals were undergoing DR.

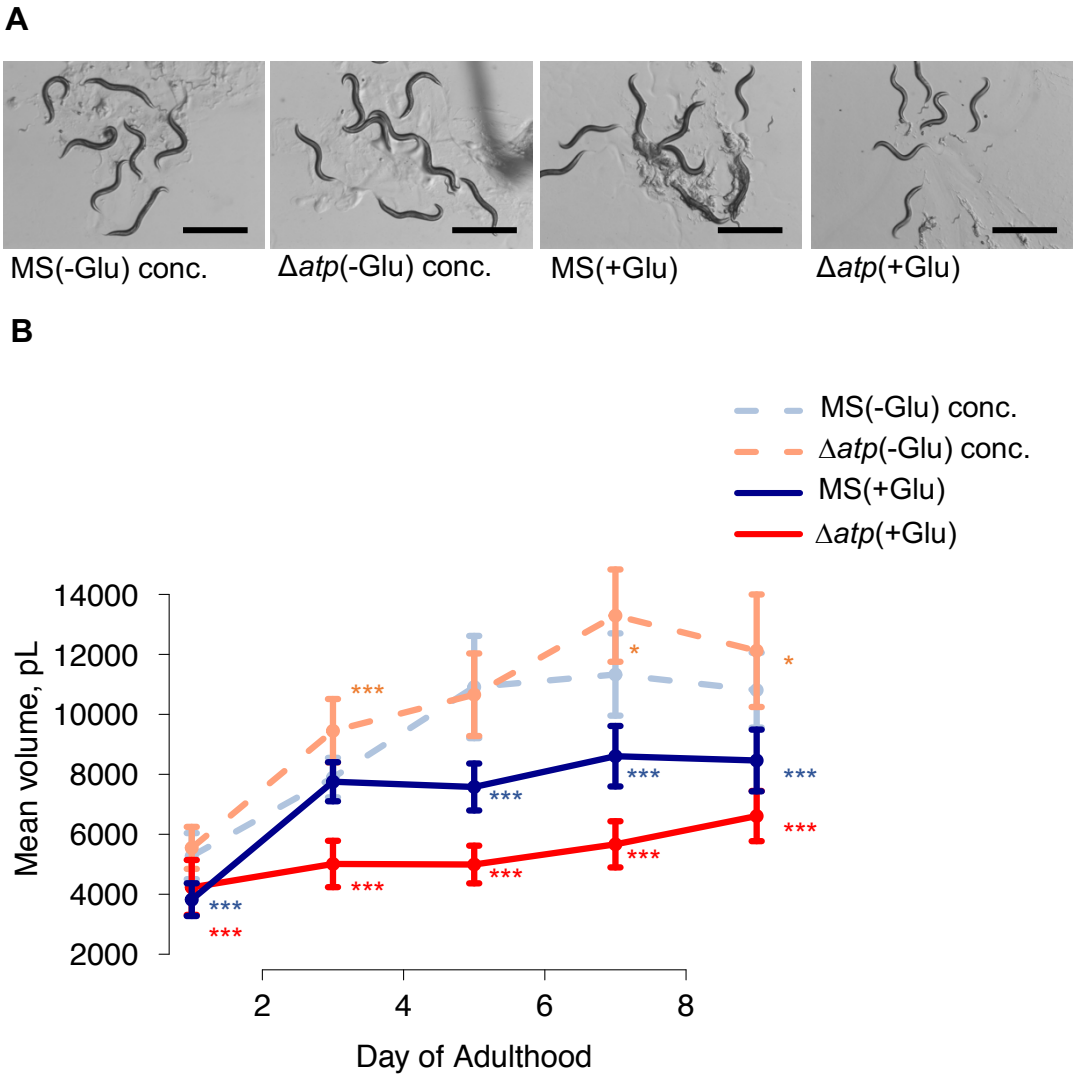



**Figure 4. 2 Body volume (picoliters, pL) of *C. elegans* was reduced by feeding on *B. subtilis* Δ*atp*(+Glu).** (A) Representative images of *C. elegans* fed on each of the diets at day 3 of adulthood taken at 1.25 x magnification. Scale bar = 1 mm. Similar images were taken on day 1, 3, 7 and 9 of adulthood. (B) Mean nematode volume (± standard deviation) at five adult timepoints (N = 57-93 nematodes). Asterisks indicate comparisons to individuals on a diet of *B. s.* MS(-Glu) on the same day of adulthood. Body size was reduced in individuals fed in the presence (+Glu) of glucose, in particular in those fed on Δ*atp*(+Glu). *, $p < 0.05$; ***, $p < 0.0001$. On each day, a Kruskal-Wallis test followed by Dunn's *post-hoc* test was used for statistical comparison.

#### 4.4.2.2 *B. subtilis* Δ*atp*(+Glu) shifted the pattern of egg laying

The effect of the diets on reproduction in *C. elegans* was investigated. A comparison of daily fecundity in days 1 - 6 of adulthood (Fig. 4. 3A) revealed a delayed schedule of egg laying when nematodes were fed on *B. s* Δ*atp*(+Glu).

When nematodes were fed on *B. s.* MS(-Glu), MS(+Glu) or Δ*atp*(-Glu), almost all progeny were laid within three days of adulthood (98.9 %, 97.1 % and 95.3 % of total progeny, respectively). However, this was not the case for those individuals fed on Δ*atp*(+Glu), where only 45.8 % of total recorded progeny were laid before day 4 of adulthood. In each case, a significant difference was observed in daily progeny count as measured by one-way ANOVA ($p < 0.0001$, day 1 and 3) or the non-parametric Kruskal-Wallis test ($p < 0.0001$, days 2 and 4). Statistics for omnibus tests as well as pairwise comparisons are given in Appendix Table B2-B4.

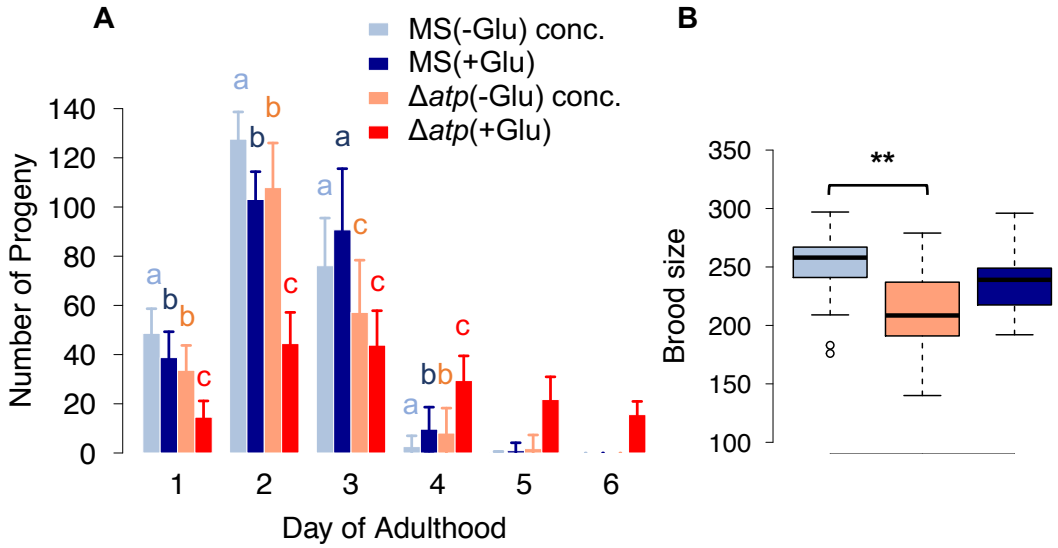



**Figure 4. 3 Nematodes fed on *B. subtilis* Δ*atp*(+Glu) had a delayed egg laying schedule.** (A) Progeny production in *C. elegans* fed on each of the *B. subtilis* diets. The progeny of 11-21 individuals were counted each day, for each condition. Bacteria grown without glucose were concentrated (conc.) prior to seeding. Error bars indicate mean ± standard deviation. For each day, bars designated with the same letter are not significantly different ($p < 0.05$). Day 1 and day 3; One-way ANOVA followed by Tukey's HSD *post-hoc* test. Day 2 and day 4; non-parametric Kruskal-Wallis test followed by Dunn's *post-hoc* (Benjamini-Hochberg FDR multiple correction). (B) Total brood size of *C. elegans* fed on each of the four *B. subtilis* diets. Feeding on *B. s.* Δ*atp*(-Glu) reduced brood size compared to *B. s.* MS(-Glu)-fed animals (**, $p = 0.005$). N= 15, 18 or 16 animals fed on MS(-Glu), Δ*atp*(-Glu) of MS(+Glu), respectively. The brood size of animals fed on Δ*atp*(+Glu) could not be determined as a result of escape prior to the end of egg laying. One-way ANOVA followed by Tukey's HSD *post-hoc* test.

When nematodes fed on *B. s.* MS(-Glu) and MS(+Glu) are compared, significantly more progeny were laid by MS(-Glu)-fed individuals on day 1 and 2 of adulthood ($p = 0.0134$, Tukey's *post-hoc* test; $p = 0.001$, Dunn's *post-hoc* test, respectively). However, on days 3 and 4 of adulthood, egg laying in *B. s.* MS(-Glu)-fed nematodes was similar to those fed MS(+Glu) and was significantly lower on

day 4 ($p$ = 0.0429, Dunn's *post-hoc* test). On days 1, 2 and 3 of adulthood, a diet of *B. s.* Δ*atp*(-Glu) led to lower progeny production compared to *B. s.* MS(-Glu) fed nematodes ($p$ < 0.0001, Tukey's *post-hoc* test; $p$ = 0.0145, Dunn's *post-hoc* test; $p$ = 0.0383, Tukey's *post-hoc* test, respectively).

However, the most striking shift in egg laying was observed in those animals fed on a diet of *B. s.* Δ*atp*(+Glu). Progeny production was lower in these animals compared to all other conditions for the first three days of adulthood ($p$ < 0.0001), with the exception of nematodes fed on *B. s.* Δ*atp*(-Glu) on day 3 ($p$ = 0.193, Tukey's *post-hoc* test). In comparison to a diet of *B. s.* MS, feeding on *B. s.* Δ*atp* reduced progeny production by 38%, 43% and 48% on days 1, 2 and 3, respectively, in the presence of glucose. On day four, however, animals fed on Δ*atp*(+Glu) laid significantly more eggs than all other conditions ($p$ < 0.001). Egg laying in nematodes fed on Δ*atp*(+Glu) was therefore reduced in early adulthood but was prolonged beyond the point at which nematodes fed on the other diets had ceased to lay.

Differences in overall brood size were also assessed (Fig. 4. 3B). The average brood size of animals fed on MS(+Glu) (236.75 ± 25.71, N=16) was not significantly different to that observed on MS(-Glu) ($p$ = 0.587, Tukey's *post-hoc* test). Interestingly, nematodes fed on *B. s.* Δ*atp*(-Glu) had a 15% reduced average brood size (210.722 ± 35.128, N=18) when compared to MS(-Glu)-fed individuals (248.267 ± 35.170, N=15) ($p$ = 0.005, Tukey's *post-hoc* test). This shows that *B. s.* Δ*atp* affects reproduction even in the absence of glucose.

High levels of escape in the *B. s.* Δ*atp*(+Glu) group were observed. From an initial sample of 27 individuals, all had escaped by the seventh day of adulthood, and as a result of this the total brood size of nematodes fed on Δ*atp*(+Glu) could not be determined. However, the average brood size ± standard deviation at the last point of observation (day 6) of individuals fed on *B. s.* Δ*atp*(+Glu) was 186.8 ± 22.332 (N=5), around 50 progeny fewer than the total brood size of animals fed on *B. s.* MS(+Glu).

Clearly, a diet of *B. s.* Δ*atp*(+Glu) led to a dramatic shift in the pattern of daily egg laying, indicating that the effects of this diet extend to reproductive fitness. This is consistent with a commonly observed phenotype of DR (Onken and Driscoll, 2010).

### 4.4.3 Was feeding of *C. elegans* on *B. s.* Δ*atp* impaired?

#### 4.4.3.1 Sporulation of *B. subtilis* MS and Δ*atp* was induced on NGM

One factor which potentially affects the edibility of *B. s.* MS or Δ*atp* is the formation of endospores (or spores herein). In order to prevent the onset of sporulation, glucose can be added to the growth medium. Excess glucose represses the activity of a response regulator which controls entry into the sporulation cycle (Dixon and Spiegelman, 2002), thereby blocking sporulation. Sporulation was previously shown to be initiated by growth on nematode growth medium (NGM), and this problem was overcome by the addition of glucose and arginine to the NGM (Gusarov *et al.*, 2013). The presence of *B. subtilis* spores has been suggested to induce DR-like phenotypes in *C. elegans* (Laaberki and Dworkin, 2008).

To clarify the extent of sporulation and its potential role in *B. s.* Δ*atp*-induced longevity, the *B. subtilis* diets were grown on NGM and screened for spore formation. Unexpectedly, spore formation was detected under all conditions (Fig. 4. 4) and seemed to be particularly prevalent in lawns of *B. s.* Δ*atp* in the absence of glucose (not quantified). In future, it will be important to quantify the extent of sporulation in each of the diets, since differential spore formation might be expected to affect *C. elegans* lifespan, and the use of spore-less bacterial diets of a different 168 background (Chapter 6) induced notable differences in the development and lifespan of *C. elegans.*

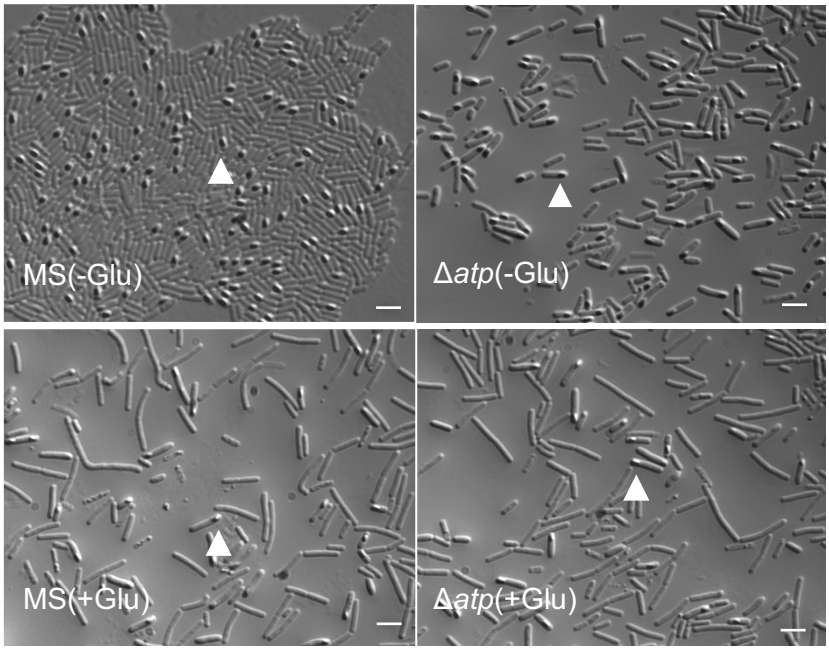



**Figure 4. 4 Spore formation in *B. subtilis* MS and Δ*atp* in the presence (+Glu) and absence (-Glu) of glucose.** Arrows indicate the phase bright/dark endospores inside the rod-shaped *Bacillus* cells. Sporulation was found to occur even in the presence of glucose. Nevertheless, *B. subtilis* forms long rods of dividing cells on NGM(+Glu) plates, showing active growth and division. Scale bar, 5 μm.

In addition to the presence of spores, one cellular property that can influence the edibility of the food is cell size. The growth rate of *C. elegans* on different bacterial species and strains shows a clear inverse correlation with increasing cell size, indicating that larger cells are more difficult to consume (Avery and Shtonda, 2003). This arises as a consequence of restrictions imposed by the diameter of the pharynx opening. Young adults of *C. elegans* are able to take in spherical beads with a diameter of 0.5 – 3 μm (Fang-Yen *et al.*, 2009). The width of *B. s.* MS and Δ*atp* grown in the absence and the presence of glucose on NGM for two days was measured (Fig. 4. 5). *B. s.* Δ*atp*(-Glu) cells were larger than *B. s.* MS(-Glu) cells in unconcentrated and concentrated lawns, and with glucose (N=103) ($p < 0.0001$, Kruskal-Wallis test with Dunn's *post-hoc* test). However, the width (< 2 μm) was not within the range that can affect cell edibility (Avery and Shtonda, 2003).

The particles are therefore able to enter the *C. elegans* pharynx. However, cells grown in the presence of glucose formed long chains (filaments). It was reasoned that, if the length of *B. s.* MS and Δ*atp* cells grown with glucose is similar,

cell length is not likely to affect edibility and be relevant for the longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu). Indeed, the average length of *B. s.* MS(+Glu) cells (6.22 ± 2.06 μm, N=183) was similar to that of *B. s.* Δ*atp*(+Glu) cells (6.62 ± 1.97 μm, N=183). The difference in cell length was not statistically significant ($p$ = 0.26, Kruskal-Wallis test with Dunn's *post-hoc* test). Cell morphology therefore does not seem to be contributing to differences in *C. elegans* physiology (Fig. 4. 5, Table B5-B6). It is possible, however, that larval feeding was affected by these size differences, and it will therefore be informative to quantify uptake in larvae.

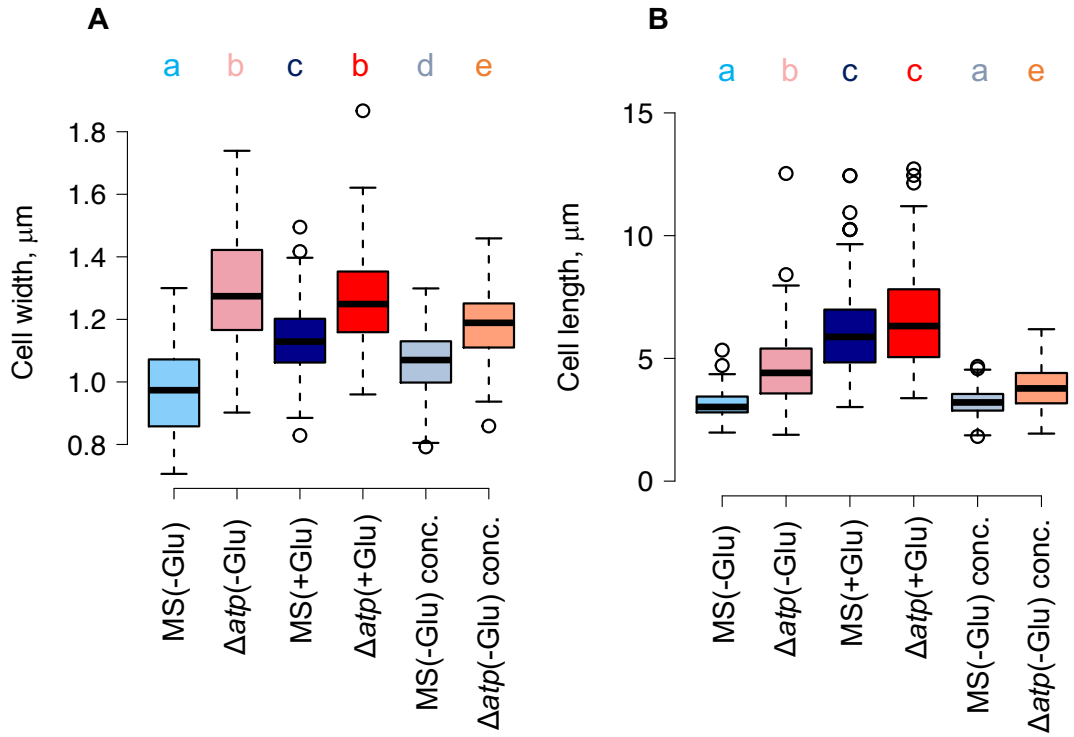



**Figure 4. 5 Cell width (A) and length (B) of *B. subtilis* MS or Δ*atp* grown in the absence and the presence of glucose.** Bacteria were seeded onto NGM(-Glu) or NGM(+Glu) and left to grow for 48 hrs before imaging and measurement. N = 103 (A), N = 183 (B). Cell size was measured in two independent trials. Different letters indicate significant differences between groups. Kruskal-Wallis test followed by Dunn's *post hoc* test. Circles depict outliers.

#### 4.4.3.2 Pharyngeal pumping was not affected by a diet of *B. s.* Δ*atp*

Caloric intake shows a linear relationship to the rate of pharynx pumping in *C. elegans* (Klass, 1977). A reduced rate of pharynx pumping therefore indicates lower food intake, and the possibility of DR if pumping is severely impaired. If *B. s.*

Δ*atp* was reducing the rate of pharynx pumping in *C. elegans,* this would restrict food intake in a way which was independent of the amount of food provided. In the previous experiments of this chapter, concentrated bacteria were provided to *C. elegans* in the absence of glucose. Following the finding that concentrated lawns up-regulated aspects of the nematode stress response (Chapter 3), unconcentrated lawns were provided to adult *C. elegans* in the absence of glucose.

Pumping rates were quantified for 20-36 individuals across two independent trials, on days 1, 4, 7 and 10 of adulthood (Fig. 4. 6). Non-parametric Kruskal-Wallis tests revealed a significant effect of diet on pharyngeal pumping on day 1 ($H = 17.98$, $p = 0.0004$), day 4 ($\chi 2 = 34.57$, $p < 0.0001$), day 7 ($\chi 2 = 32.73$, $p < 0.0001$) and day 10 ($\chi 2 = 20.17$, $p = 0.0002$) of adulthood. Pairwise comparisons between groups on each day were made using Dunn's *post-hoc* test (false discovery rate *p* value correction). A number of individuals were outside of the bacterial lawn for a part or the majority of the one-minute observation period (encircled outliers), and these were excluded from statistical analysis. See Appendix Table B7 for statistical comparisons.

Unexpectedly, the rate of pharynx pumping was lowest in individuals fed on *B. s.* MS(-Glu) throughout adulthood ($p < 0.05$). In comparison to individuals fed on *B. s.* MS(+Glu), median pumping rate was reduced by 5% ($p = 0.0133$), 7% ($p = 0.0119$), 12% ($p = 0.0002$) and 38% ($p = 0.099$) on days 1, 4, 7 and 10 of adulthood, respectively. Moreover, a decline in the rate of pharynx pumping was most noticeably observed in animals fed on *B. s.* MS(-Glu), possibly reflecting a higher rate of ageing-associated deterioration in function. By day 10, median pumping rate of these individuals was only 167.5 $min^{-1}$, about 40% lower than animals on the other diets. The outliers at day 7 and 10 in Fig. 4. 6 correspond to animals in which pharynx pumping had slowed or ceased, which is known to occur in middle-aged and old individuals (Chow *et al.*, 2006). A large proportion of the *B. s.* MS(-Glu) population were in a state of decline at day 10, as reflected in the wide, evenly spread distribution of data points.

A diet of *B. s.* Δ*atp*(+Glu) did not significantly reduce pharynx pumping rate in comparison to a diet of *B. s.* MS(+Glu) on day 1 ($p = 0.9802$), day 4 ($p = 0.2197$), day 7 ($p = 0.0817$) or day 10 ($p = 0.0721$) of adulthood. However, 5 animals out of 27 on day 1 and 3 out of 26 on day 4, were found outside of any bacteria for a large

proportion of the observation time, and as a result the pumping was greatly lowered (encircled outliers, Fig. 4. 6). These data reveal that, discounting animals that roamed the bare agar away from food, *B. s.* Δ*atp*(+Glu) did not reduce the rate of pharyngeal pumping. Strikingly, nematodes fed on *B. s.* Δ*atp*(-Glu) had a high rate of pharynx pumping on each day. Median pumping rate in these animals was consistently higher than those fed *B. s.* MS(-Glu) on day 1 (9%>, $p = 0.0002$), day 4 (12%>, $p < 0.0001$), day 7 (17%>, $p < 0.0001$) and day 10 (66%>, $p = 0.0006$).

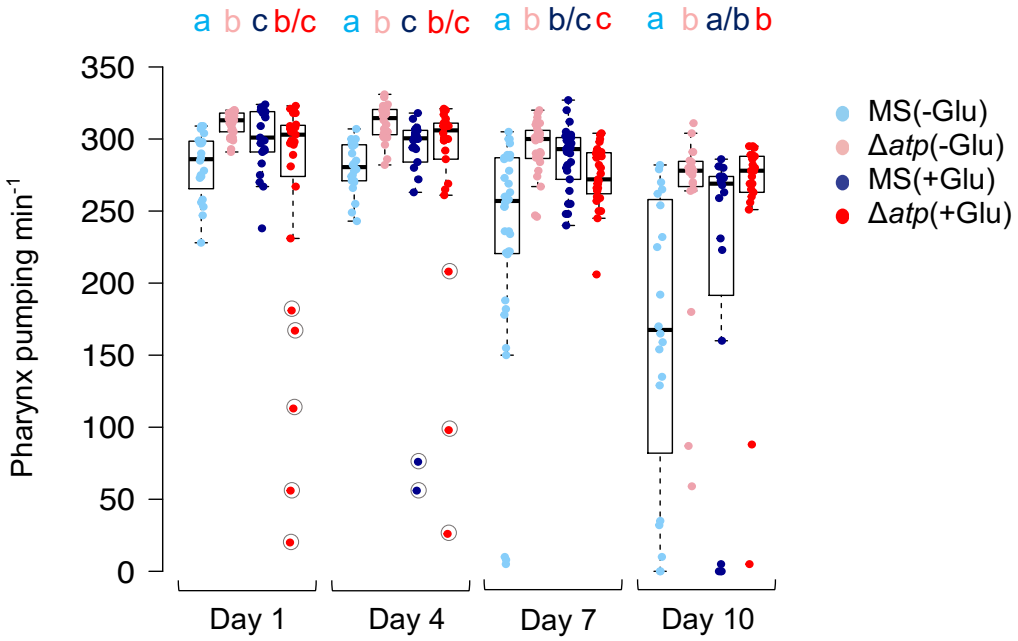



**Figure 4. 6 *B. s.* Δ*atp*(+Glu) did not reduce the pharyngeal pumping rate of *C. elegans*.** The number of pumps per minute was quantified in a total of 20-36 animals across two independent trials. Outliers surrounded by circles indicate animals that were outside of bacteria during the observation period. These were excluded from statistical analysis. Data were analysed using a non-parametric Kruskal-Wallis test followed by Dunn's *post-hoc* test with FDR correction of *p* values. Different letters indicate significant differences between diets on the same day ($p < 0.05$).

#### 4.4.3.3 Bacterial lawn morphology was altered by deletion of ATP synthase

Aside from cell size and pharyngeal pumping rate, another factor that could influence food edibility is the physico-chemical properties of lawn growth. There were clear morphological differences between lawns of *B. s.* MS and *B. s.* Δ*atp* after growth at 20 °C, as shown in Fig. 4. 7. Growth of *B. s* Δ*atp*(-Glu) was reduced considerably, but an increased formation of small, hard areas of matrix was apparent over the lawn. Both *B. s.* MS(+Glu) and *B. s.* Δ*atp*(+Glu) developed a thick

extracellular matrix. However, the growth of the Δ*atp* matrix was more pronounced than that of MS. In particular, the central region of the mutant lawn was quickly overtaken by matrix growth, whilst matrix growth of the MS was confined mostly to the border of the lawn (Fig. 4. 7A).

Several observations related to nematode morphology and behaviour indicated that these differences in lawn growth might be influencing the lifespan of *C. elegans*. As discussed in Chapter 3, larvae fed on *B. s*. Δ*atp* were developmentally delayed, and were particularly asynchronous when glucose was present. Nematodes introduced onto Δ*atp*(+Glu) lawns escaped in relatively high numbers during the previous lifespan experiments. Animals fed on Δ*atp*(+Glu) were more commonly found outside of bacteria during the measurement of pharyngeal pumping (Fig. 4. 6). Similarly, as shown in Fig. 4. 7B, larvae introduced onto these lawns were often found outside of the central lawn. On the other diets, *C. elegans* remained in the central lawn.

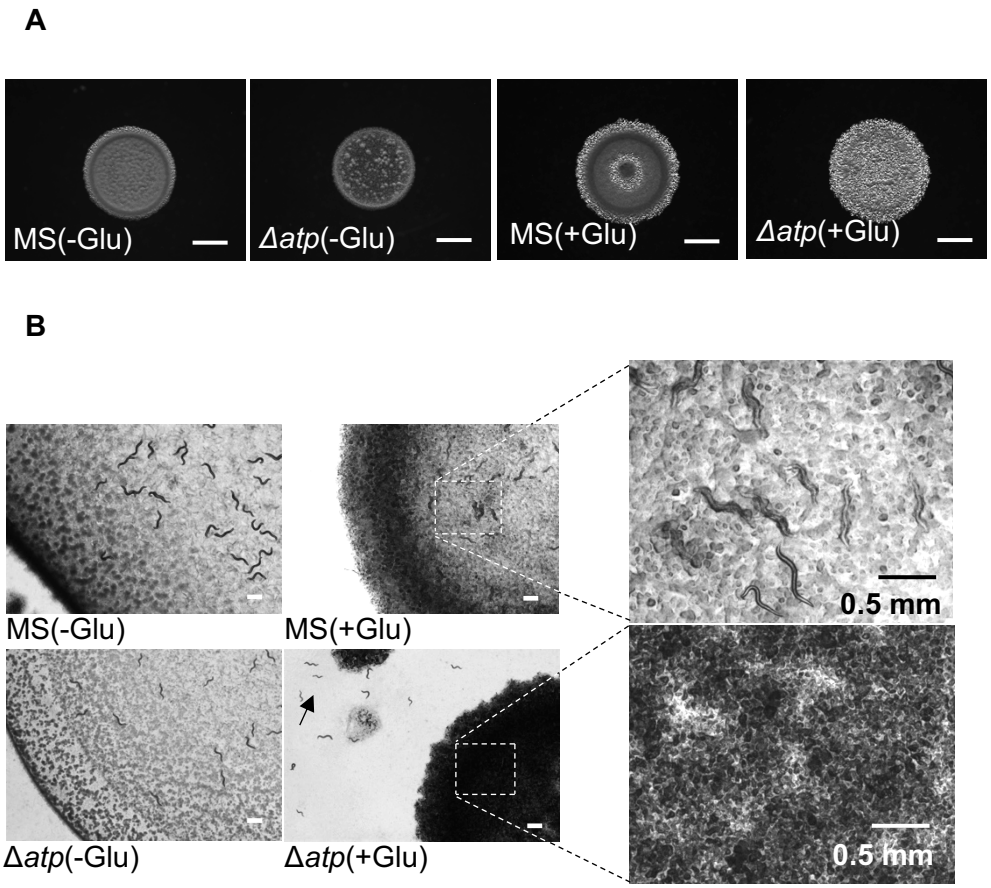



**Figure 4. 7 *B. subtilis* lawn morphology was altered by deletion of ATP synthase.** (A) Comparison of the lawn morphology of *B. subtilis* MS and Δ*atp* in the absence (-Glu) and the presence (+Glu) of glucose. Bacteria were not concentrated. Darkfield images were taken at 7 x magnification. 2 µl of each lawn were seeded onto NGM(-Glu) or (+Glu) plates and incubated at 20°C for 56 hours. *B. s.* Δ*atp* had more extensive growth of the extracellular matrix over the entire surface of the lawn in the presence of glucose. Scale bar = 1 mm. (B) *C. elegans* larvae fed on *B. s.* Δ*atp*(+Glu) lawns rarely resided within areas of substantial growth. Images were taken 48 hrs after introduction of L1s onto the indicated diet (10 x magnification) (3 day's lawn growth at 20 °C). Lawn images show that larvae fed on *B. s.* MS(-Glu), MS(+Glu) and Δ*atp*(-Glu) were located within the central lawn and were rarely found outside. Bacteria were not concentrated. In the presence of glucose, the growth of an extracellular matrix was apparent. However, whilst *B. s.* MS(+Glu) lawns remained largely loose, *B. s.* Δ*atp*(+Glu) lawns became largely engulfed by a dry matrix. Enlarged sections of lawns in the presence of glucose demonstrate that animals tended to reside within the central MS(+Glu), but not in the central *B. s.* Δ*atp*(+Glu) lawn. Instead, larvae were found around smaller patches of *B. s.* Δ*atp*(+Glu) growth (black arrow). Scale bar = 500 µm.

It is important to note that the lawn growth of both strains with glucose after 24 hrs (the timepoint at which nematodes were transferred onto them) was

considerably less extensive than in Fig. 4. 7. An extracellular matrix had not formed over the surface of either lawn at this point (Fig. 4. 8).

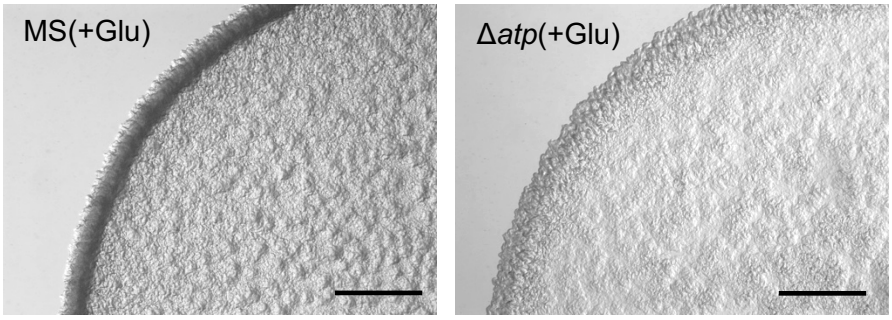



**Figure 4. 8 Lawn growth of *B. s*. MS and Δ*atp* on NGM(+Glu) 24 hours after seeding (the point at which nematodes were introduced).** Matrix growth was minimal by this timepoint and the lawns of both strains were loose. Scale bar = 1 mm.

#### 4.4.3.4 *C. elegans* resided predominantly within thinner areas of *B. s.* Δ*atp*(+Glu) growth

A combination of *B. s.* Δ*atp* and glucose appeared to be a requirement for this diet to elicit substantial longevity in *C. elegans*. One explanation for this is that metabolites differentially produced by Δ*atp*(+Glu) were responsible for extending lifespan. Another possibility is that matrix overgrowth of the *B. s.* Δ*atp*(+Glu) lawn induced DR by making the bacteria inaccessible to *C. elegans.* Previously, the quality of the food source – defined as its ability to support development of *C. elegans* – was shown to regulate *C. elegans* locomotion (Shtonda and Avery, 2006). Low quality diets (including *B. megaterium*) were hard to eat as a consequence of large cell size (Avery and Shtonda, 2003), and stimulated increased roaming behaviour of *C. elegans,* whilst the opposite was true for high quality diets (e.g. *Comamonas*). *C. elegans* shows a preference for higher quality diets in food choice assays (Shtonda and Avery, 2006). Increased avoidance of food extends *C. elegans* lifespan whilst reducing fecundity and fat storage (Lee and Mylonakis, 2017).

To investigate whether *C. elegans* avoided *B. s.* Δ*atp*(+Glu) *per se,* or perhaps showed a preference for certain types of bacterial growth, the proportion of nematodes outside of the bacterial lawn and in different regions of bacterial growth was quantified (Fig. 4. 9). Data were collected on days 1, 4, 7, 10 and 14 of

adulthood by another investigator (Neilson, 2017). The bacterial lawns were categorised into 4 'zones' as follows: (1), the central bacterial lawn; (2) the border of the bacterial lawn which was slightly looser in consistency than the centre; (3) the outer edge of the bacterial lawn, and patches of growth outside of the area where the plates were seeded; (4), bare unseeded NGM with no growth. Zones 1, 2 and 3 therefore represent different types of bacterial growth, whereas zone 4 is the absence of food. Following seeding of bacteria and growth of the lawn for 24 hrs, only zones 1, 2 and 4 existed on the agar plates. Zone 3 arose as a consequence of roaming behaviour, which spread bacteria over the surface of the NGM and lead to patches of thinner growth across the plate. See Appendix Table B8 and B9 for summary data and statistical comparisons.

Here, again, bacteria were not concentrated prior to seeding in the absence of glucose (the experiment was conducted concomitantly with the lifespan assay presented in Fig. 3. 4, Chapter 3, in which the effect of unconcentrated bacteria on lifespan was investigated). However, all nematodes developed in the absence of glucose on concentrated lawns of the respective diet.

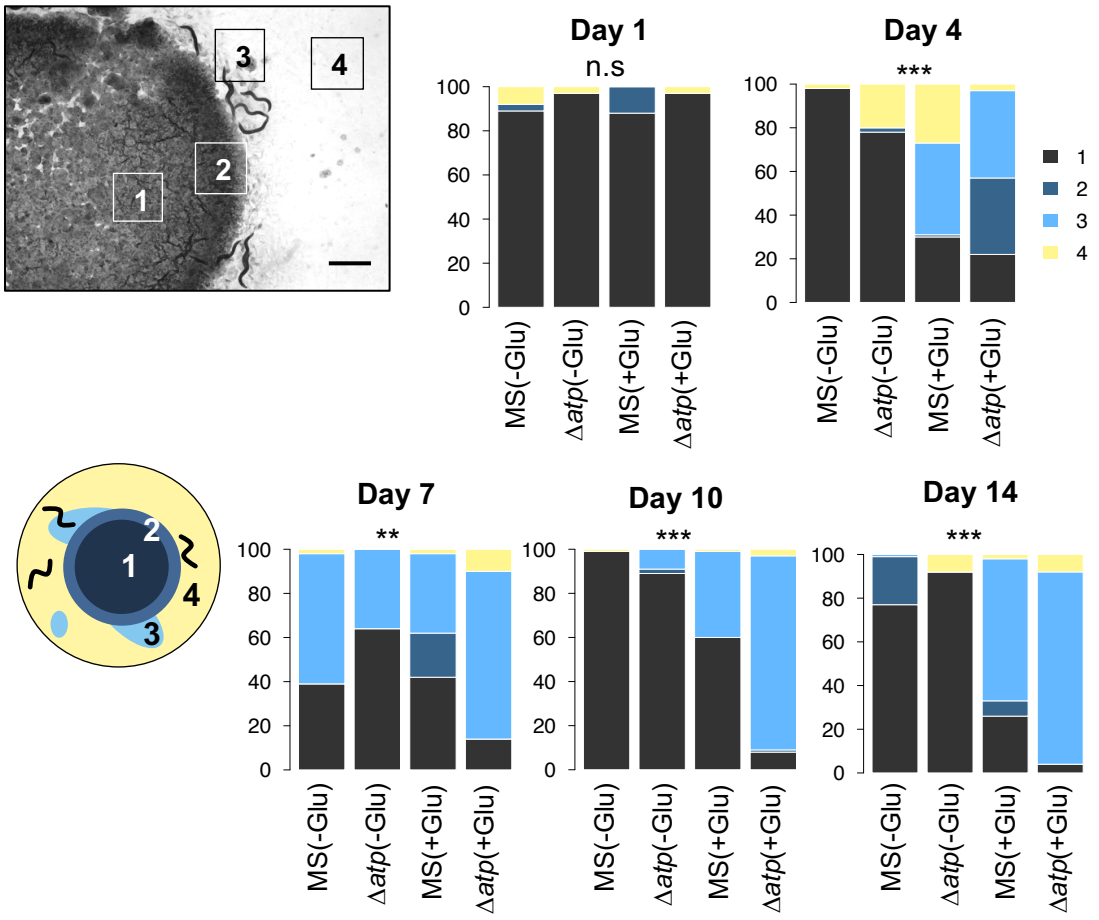



**Figure 4. 9 Quantification of *C. elegans* location on lawns of *B. s.* MS and Δ*atp*.** Four zones were defined based on the growth of the bacteria on the NGM. Zone 1, central lawn; zone 2, lawn border (often thicker and 'looser' than zone 1); zone 3, thinner growth spread by nematode migration; zone 4, no bacterial growth. Depicted is a lawn of *B. s.* MS in the presence of glucose. Day 5 adults are shown. Scale bar = 1mm. Stacked box plots depict the location of nematodes inside the different zones. Values are given as percentages of total individuals in each condition. N=40 animals per condition per day. A large proportion of individuals fed on *B. s.* Δ*atp*(+Glu) were in zone 3 from day 4 onwards. Asterisks indicate a significant association between diet and lawn location. **, $p < 0.01$; ***, $p < 0.0001$, Pearson's Chi-square test. Data were collected by (Neilson, 2017).

Within each day, Pearson's Chi-square test was used to test the association between diet and nematode location on the agar plate (N = 40 animals per condition per day). On day 1, there was no significant association ($p = 0.896$). Most nematodes were located inside the central lawn on each of the diets. However, diet had a significant effect on lawn location on all subsequent days ($p < 0.0001$). With the exception of day 7, a majority of nematodes fed in the absence of glucose remained within the central lawn.

A striking trend was the tendency of nematodes fed on *B. s.* Δ*atp*(+Glu) to reside inside thinner areas of bacterial growth around the edge of the central lawn (zone 3). On days 4, 7, 10 and 14, the proportion of animals in this type of growth was 40%, 76%, 88% and 88%, respectively. The proportion of nematodes fed on *B. s.* MS(+Glu) in zone 3 was similarly high (~ 40% on days 4-10, and 65% on day 14). Although these nematodes were located outside of the central lawn zone, they were very rarely observed outside of any bacteria at all (zone 4). In the presence of glucose on each day, 10% or less of nematodes were found in zone 4, with the exception of *B. s.* MS(+Glu) on day 4 (27%). In fact, a higher percentage of animals fed on *B. s.* Δ*atp* were found in zone 4 when glucose was not present (20% on day 4, <10% subsequently). It can be concluded from these observations that animals fed on *B. s.* Δ*atp*(+Glu) were not avoiding bacterial growth *per se* but preferred thinner areas of the lawn growth over the matrix-encrusted central lawn.

### 4.4.4 Chloramphenicol abolished the longevity of *B. s.* Δ*atp*(+Glu)-fed nematodes

The lawn location data suggested that most nematodes avoided bacterial overgrowth but were found within bacteria on the NGM plates. If matrix overgrowth was not limiting the availability of food for *C. elegans,* then preventing excessive growth and matrix formation should not alter the extent of longevity. Chloramphenicol (0.7 μg/ml) was added to the NGM to slow the rate of bacterial lawn growth and prevent excess matrix formation (Fig. 4. 10). A matrix was not visible on lawns of *B. s.* MS or Δ*atp.*

In the presence of this antibiotic, the bacterial lawn still grew visibly thicker over the course of two days, showing that the bacteria were still capable of division and were metabolically active (Fig. 4. 10A). The growth of the lawn was sufficient to sustain the animals, and no escape was observed over the course of the experiment (see Appendix Table B10). Strikingly, the presence of chloramphenicol completely abolished the longevity of Δ*atp*(+Glu)-fed individuals (Fig. 4. 10B). With the addition of chloramphenicol, the lifespan of *C. elegans* fed on *B. s.* MS(+Glu) (N=129) or Δ*atp*(+Glu) (N=129) was not significantly different ($p$ = 0.337, log rank test). However, individuals fed Δ*atp*(-Glu) (N=114) had a 13% increase in mean lifespan compared to those fed *B. s.* MS(-Glu) (N=127) ($p$ < 0.0001, log rank test). Interestingly, glucose became detrimental to nematodes fed on *B. s.* MS when chloramphenicol was present ($p$ <0.0001, log rank test). Also, for the first time

without FUdR, the mutant was observed to increase lifespan without glucose being present, by 13% ($p < 0.0001$, log rank test). *C. elegans* was observed to remain within the bacterial lawn of each diet, and body size was similar in each case, without the marked size reduction in animals fed on *B. s.* Δ*atp*(+Glu) that was recorded previously (not quantified).

This experiment demonstrates that, rather than a negative effect of *B. s.* MS(+Glu), a pro-longevity action of *B. s.* Δ*atp*(+Glu) was responsible for increasing the lifespan of *C. elegans*. Moreover, glucose was only beneficial to *C. elegans* fed on *B. s.* Δ*atp* when the bacteria were proliferating abundantly, and not in a growth-impaired state. This indicates that, rather than a glucose-induced metabolite, some aspect of abundant lawn growth was responsible for eliciting longevity.

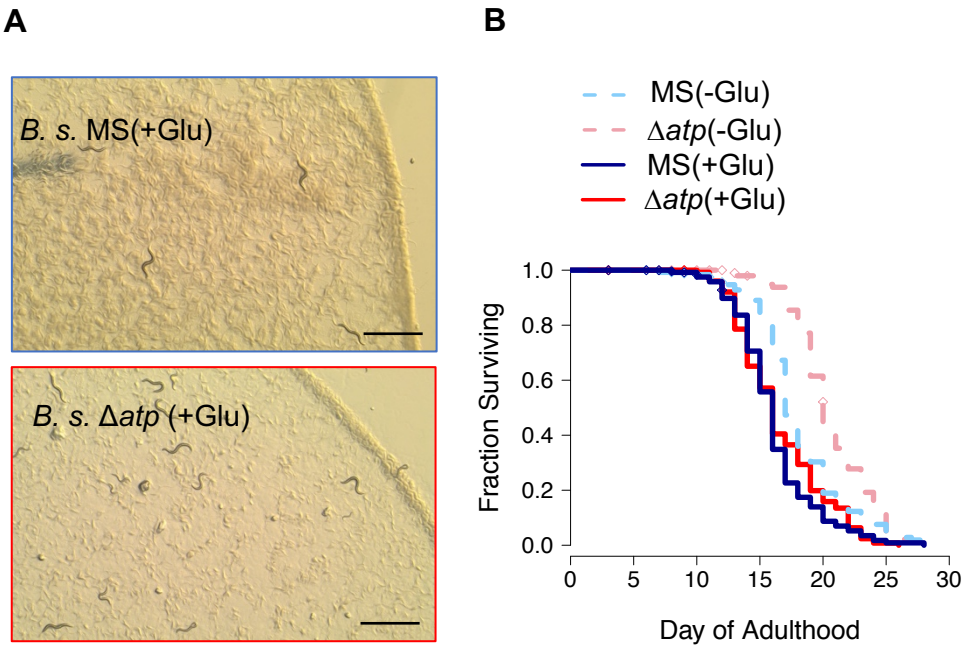



**Figure 4. 10 Chloramphenicol altered lawn morphology (A) and abrogated the longevity of nematodes fed on *B. s.* Δ*atp*(+Glu) (B).** (A) Chloramphenicol (0.7 μg/ml) slowed lawn growth and altered lawn morphology of both diets. The formation of a matrix was no longer apparent on either diet, even when glucose was present (as depicted). L4 stage nematodes can be seen on both lawns, which were introduced onto the plates two days prior to imaging. The lawns had been growing for four days at 20 °C at the time of imaging. Scale bar = 1mm. (B) Lifespan of *C. elegans* fed on *B. s.* MS or Δ*atp* in the presence of 0.7 μg/ml chloramphenicol. The lifespan of *C. elegans* fed on *B. s* Δ*atp*(+Glu) (N=129) was not significantly different to that of animals fed on *B. s.* MS(+Glu) (N=129) ($p = 0.337$). However, *B. s.* Δ*atp*(-Glu) extended lifespan (N=114) in comparison to animals fed on *B. s.* MS(-Glu) (N=127) ($p < 0.0001$). The experiment was conducted at 20 °C. Diamond marks represent censoring. The log rank test with Holm-Sidak test for multiple comparisons was used for pairwise comparisons between conditions.

### 4.4.5 Does *B. s.* Δ*atp* regulate DR pathways in *C. elegans*?

#### 4.4.5.1 Mutation of *eat-2* and a diet of *B. s.* Δ*atp*(+Glu) additively extended *C. elegans* lifespan

As discussed above, several lines of evidence indicate that DR is involved in the longevity of animals fed on *B. s.* Δ*atp*(+Glu). This hypothesis was directly tested by measuring the lifespan of *C. elegans* strain *eat-2(ad1116)* when fed on *B. s.* MS(+Glu) or Δ*atp*(+Glu) (Fig. 4. 11). The low rate of pharynx pumping in *eat-2* individuals induces constitutive DR. If the long lifespan of *eat-2(ad1116)* nematodes is unaffected (not further extended) by a diet of *B. s.* Δ*atp*(+Glu), this would offer strong evidence that DR is responsible for the longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu). Alternatively, if a diet of *B. s.* Δ*atp*(+Glu) extends lifespan of *eat-2(ad1116)* individuals to a considerable degree, and particularly if this increase is similar to that observed for N2 worms, this would indicate that *B. s.* Δ*atp* and *eat-2* mutation extend lifespan through distinct mechanisms. Such an observation would leave open the possibility that DR does not account for mutant-induced longevity.

The *eat-2(ad1116)* strain was first confirmed to have a low rate of pharyngeal pumping (consistently below 60 pumps $min^{-1}$) (Fig. 4. 11A). A diet of *B. s.* Δ*atp*(+Glu) did not affect the pharyngeal pumping rate on any of the days of adulthood tested ($p > 0.05$, two-sample t test) (Appendix Table B11-B12). Larvae were again fed in the absence of glucose (concentrated lawns), and *eat-2(ad1116)* animals had a developmental delay compared to N2 as has been previously reported for this strain. Whether there was a further delay elicited by feeding on *B. s.* Δ*atp*(-Glu), as for N2, was difficult to discern and will require closer staging of larvae.

As can be seen in Fig. 4. 11B, *eat-2(ad1116)* individuals were longer-lived than N2 individuals, whether they were fed on *B. s.* MS (N=136) or Δ*atp* (N=144) ($p < 0.0001$, log rank test). Moreover, feeding on *B. s.* Δ*atp*(+Glu) increased lifespan to a similar extent in N2 (17% >) and *eat-2* (19% >) nematodes, compared to individuals fed *B. s.* MS(+Glu) ($p < 0.0001$, log rank test). Longevity afforded by *B. s.* Δ*atp* and *eat-2* was therefore additive, indicating that *B. s.* Δ*atp*(+Glu) and mutation of *eat-2* increased lifespan by distinct mechanisms. Strikingly, 18 *eat-2(ad1116)* animals fed on *B. s.* Δ*atp*(+Glu) escaped from the assay plates during the experiment, compared to only 3 individuals fed on MS(+Glu), as shown in

Appendix Table B13, and roaming of these animals across the plate was elevated. The lawn preference of N2 animals in each condition was similar to that depicted in Fig. 4. 9 (not quantified).

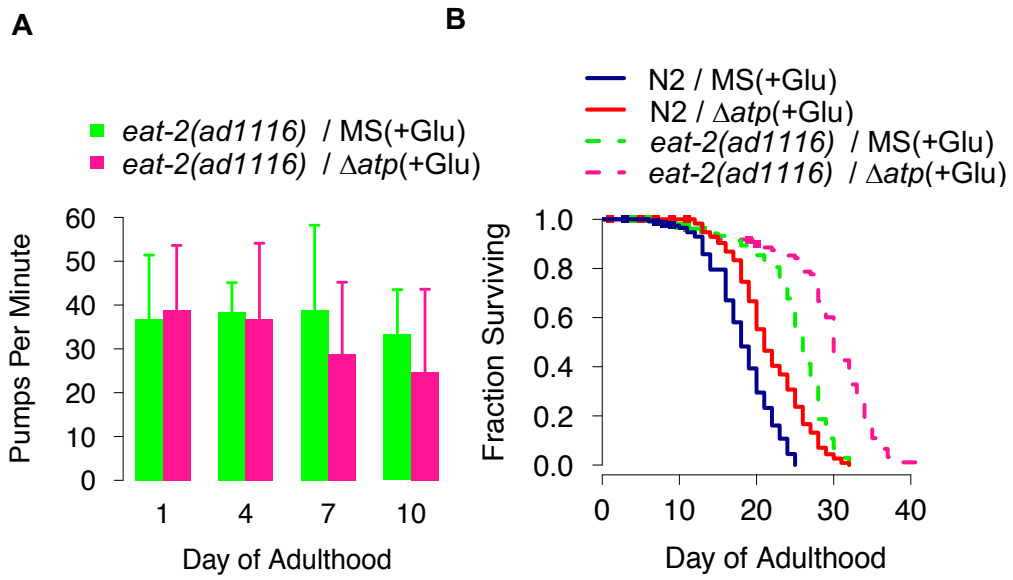



**Figure 4. 11 A diet of *B. s.* Δ*atp*(+Glu) extended the lifespan of feeding-defective *eat-2(ad1116)* animals without affecting pharyngeal pumping.** (A) Pumping rate per minute of *eat-2(ad1116)* animals fed on *B. s.* MS(+Glu) or Δ*atp*(+Glu). On each day, diet did not affect pumping rate ($p > 0.05$). The data also confirmed that *eat-2(ad1116)* individuals had a low rate of pharynx pumping (< 60 $min^{-1}$), which leads to dietary restriction and longevity in this strain. Pumping rates of 10 individuals on each day were compared with a two-sample t test. (B). *B. s* Δ*atp* increased the lifespan of N2 *C. elegans* and *eat-2(ad1116)* mutant to a similar extent, in the presence of glucose. *eat-2(ad1116)* nematodes are longer-lived than N2, when either *B. s*. MS or Δ*atp* are provided as food ($p < 0.0001$, log rank test). The lifespan of long-lived *eat-2(ad1116)* individuals was further extended by Δ*atp* compared to *eat-2(ad1116)* fed on MS ($p < 0.0001$, log rank test). All experiments were undertaken in the presence of 2% glucose (+Glu).

#### 4.4.5.2 *B. s.* Δ*atp* altered DR-associated gene expression in *C. elegans*

In order to begin to understand the signalling pathways in *C. elegans* that were being modulated by the *B. subtilis* diets, and to test the hypothesis that *B. s.* Δ*atp*(+Glu) induced a DR-like state in *C. elegans,* the expression of a set of genes was monitored in day 2 adults.

These genes included regulators of IIS – the insulin-like receptor *daf-2,* the FOXO transcription factor *daf-16* and the *daf-16* target gene *sod-3* – as well as TOR/steroid hormone signalling pathways (*let-363*/TOR and the cytochrome P450

enzyme *daf-9*). The SKN-1 and PHA-4 transcription factors, too, play a key role in several DR regimes, and the expression of *bec-1,* which is regulated by TOR signalling and PHA-4, is important for autophagy-mediated lifespan extension by DR (Hansen *et al.*, 2008). Finally, the expression of HSF-1 and the alpha-subunit of the energy-sensing kinase AMPK (*aak*-2) were measured (Greer *et al.*, 2007). The internal controls used for normalisation of gene expression changes were *pmp-3,* which encodes a peroxisomal membrane protein, and *act-1,* which encodes actin. In one study, these genes – in particular *pmp-3* – were found to be amongst the most stably expressed in a set of *C. elegans* internal control candidates (Zhang *et al.*, 2012). The expression of ageing-associated genes normalised to the internal control gene *pmp-3* is given in Fig. 4. 12. Plots normalised to *act-1* are shown in Appendix Fig. B1. Raw data and statistical comparisons are given in Appendix Table B14 and B15.

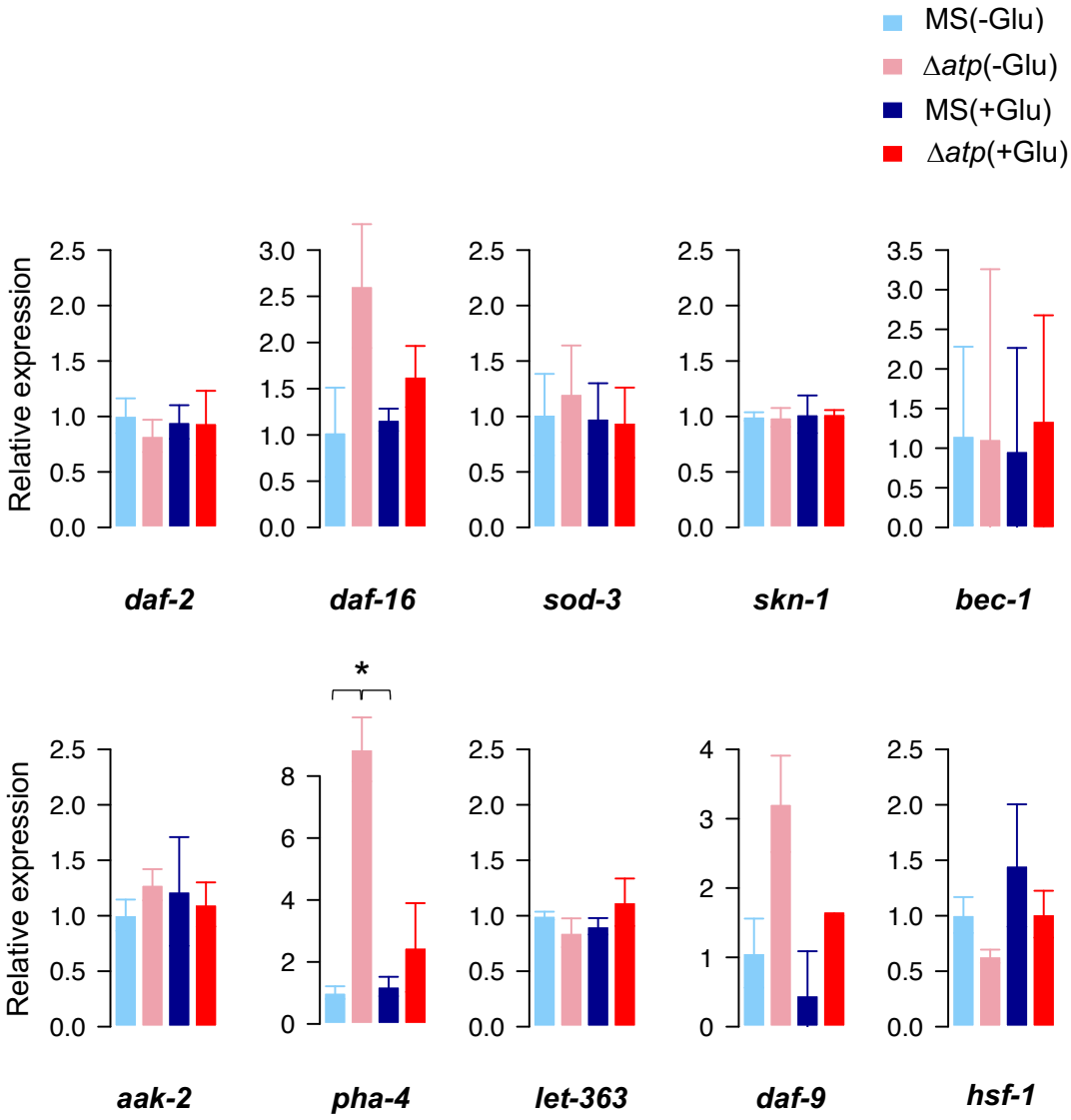



**Figure 4. 12 qRT-PCR expression analysis of genes involved in IIS signalling, TOR signalling and DR.** Expression was measured in day 2 adult *C. elegans* raised on each of the *B. subtilis* diets and expressed as 2^-ΔΔCt values relative to *B. s.* MS(-Glu) (N = 2 biological replicates). Animals fed on *B. s.* Δ*atp*(-Glu) displayed a distinct upregulation of *pha-4* expression (mean ΔΔCt = -3.14 (*pmp-3*), -2.37 (*act-1*) normalised to *B. s.* MS(-Glu)). Bars show mean ± standard deviation of relative expression. ΔCt values were calculated relative to *pmp-3* expression and were compared using ANOVA and Tukey's *post-hoc* test. *, $p < 0.05$.

Statistical comparisons were made on raw ΔCt values as opposed to relative expression values. ΔΔCt values were calculated relative to animals fed on *B. s.* MS(-Glu). Diet did not affect the expression of the majority of genes tested (one-way ANOVA, $p > 0.05$). However, there was some indication that *daf-16* expression

was elevated in *C. elegans* fed on *B. s.* Δ*atp*(-Glu), although this was not significant (mean ΔΔCt = -1.31 (*pmp-3*), -0.45 (*act-1*) $p = 0.137$ and 0.92*,* respectively).

However, a diet of *B. s.* Δ*atp*(-Glu) elicited fairly dramatic changes in gene expression. The expression of the DR-regulated gene *pha-4* was increased in these individuals by 5- to 9-fold in comparison to animals fed on *B. s.* MS(-Glu) (mean ΔΔCt = -3.14 (*pmp*-3), -2.372 (*act*-1)). The expression of *pha-4* in *C. elegans* fed on *B. s.* Δ*atp*(+Glu) was ~3-fold lower than, but not significantly different to, expression in the absence of glucose ($p = 0.0596$, *pmp-3*), and was ~2-fold higher than in animals fed on MS. This indicates, therefore, that *pha-4* levels were also elevated in animals fed on Δ*atp*(+Glu) (mean ΔΔCt = -1.17 (*pmp-3*), -0.72 (*act-1*)).

The expression of *daf-9* was also higher in *C. elegans* fed on *B. s.* Δ*atp*(-Glu), although expression was not significantly different across diets (mean ΔΔCt = -1.665 (*pmp*-3), -0.87 (*act*-1), p = 0.0753 and 0.1952, respectively). Revealingly, *let-363/*TOR, a regulator of DAF-9, was downregulated in these animals when *act-1* was used for normalisation (mean ΔΔCt = 4.694 relative to MS(-Glu), $p < 0.05$, Appendix Fig. B1). *C. elegans* fed on *B. s.* Δ*atp*(+Glu) had a similar, if lower magnitude, upregulation of *daf-9* (mean ΔΔCt = -0.7 (*pmp-3*), -0.26 (*act-1*)), though no changes in *let-363* expression were apparent. More biological replicates will however be required to increase the statistical power of the analysis. The expression of *hsf-1* was also consistently lower in animals fed on *B. s.* Δ*atp*(-Glu) (mean ΔΔCt = 0.66 (*pmp*-3), 2.0 (*act*-1)), corresponding to a 2- to 3-fold change decrease in expression.

Overall, the expression profiles of *C. elegans* fed on *B. s.* Δ*atp* indicate that key DR-related genes are regulated by this diet. Unexpectedly, *pha-4* upregulation, *let-363* downregulation and *daf-9* upregulation, which are seen in diet-restricted nematodes (Greer *et al.*, 2007; Thondamal *et al.*, 2014), were more apparent in *C. elegans* fed on *B. s.* Δ*atp*(-Glu).

As mentioned, *B. s.* Δ*atp* forms a more extensive extracellular matrix, indicating rudimentary biofilm formation. The biofilms of species including *B. subtilis, Lactobacillus rhamnosus*, *Pseudomonas fluorescens* are beneficial for *C. elegans*, and exert their benefits inside the nematode intestine by inducing stress responses in *C. elegans.* The metallothionein *mtl-1* and the heat shock protein *hsp-70* were

recently shown to be upregulated by biofilm and were required for biofilm-mediated effects on lifespan and stress responses. The expression of these genes was therefore measured (Fig. 4. 13). There was no upregulation of either *mtl-1* or *hsp-70* by *B. s.* Δ*atp*. In fact, the expression of *hsp-70* was approximately two-fold higher in animals fed on *B. s.* MS(-Glu) compared to other diets ($p < 0.05$), although this was only true using *act-1,* but not *pmp-3,* for normalisation.

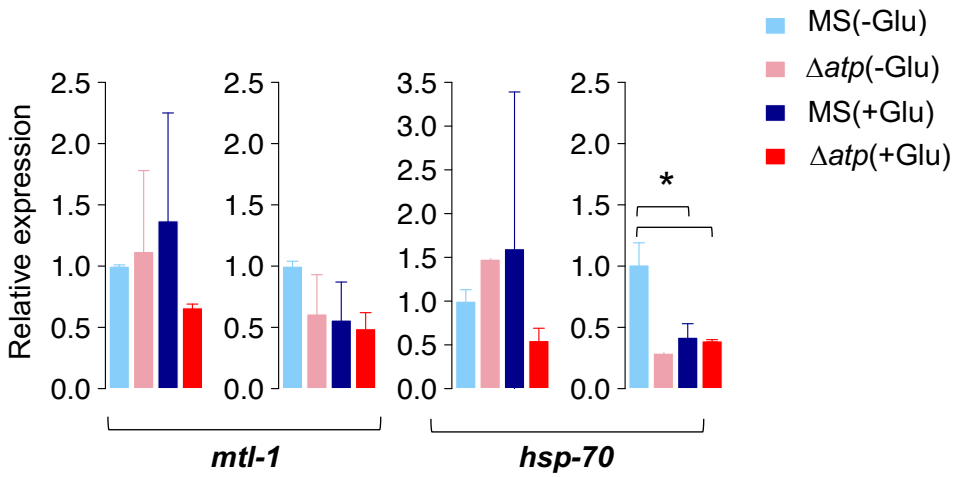



**Figure 4. 13 qRT-PCR expression analysis of *C. elegans* genes involved in the response to biofilm formation.** Depicted are 2^-ΔΔCt values normalised to *B. s.* MS(-Glu) using *pmp-3* (left) or *act-1* (right) as internal controls, for both *mtl-1* and *hsp-70*. N = 2 biological replicates for each gene, except for *hsp-70* expression in *C. elegans* fed on *B. s.* Δ*atp*(-Glu) (N = 1). Bars show mean ± standard deviation of relative expression. ΔCt values were compared using ANOVA and Tukey's *post-hoc* test. *, $p < 0.05$.

## 4.5 Discussion

### 4.5.1 A diet of *B. s.* Δ*atp*(+Glu) induced phenotypes associated with DR

In this chapter, the role of dietary restriction in Δ*atp*(+Glu)-induced longevity was investigated. Indeed, the data presented demonstrate that certain phenotypes of *C. elegans* fed on this diet are consistent with DR.

#### 4.5.1.1 Body size was reduced by *B. s.* Δ*atp*(+Glu)

DR typically induces a reduction in body size in a number of organisms including *C. elegans* (Lenaerts *et al.*, 2008). When *C. elegans* were fed on Δ*atp*(+Glu), their body size was considerably reduced, consistent with DR. However, food composition, as well as quantity, can have a marked effect on *C. elegans* physiology. When *C. elegans* were fed on the *E. coli* strain HB101, its body size was 1.6 times larger than that of animals fed on *E. coli* OP50 (So *et al.*, 2011). A change in macronutrient composition between HB101 and OP50, including a 3-5 x increase in carbohydrate levels in HB101 (Brooks *et al.*, 2009), is proposed to underlie these changes. It is not currently known whether macronutrient composition is altered by deletion of ATP synthase. However, deletion of this gene is likely to effect broad changes in bacterial metabolism, and therefore a change in nutrient composition might be expected. Interestingly, this effect must depend on a shift in nutrient composition elicited by growth in glucose. A comparison of macronutrient and micronutrient composition of *B. s.* MS and Δ*atp* in the absence and presence of glucose would therefore be potentially revealing.

Importantly, changes in body size that arise through differences in dietary nutrient composition do not necessarily correlate with nematode lifespan; animals fed on *E. coli* HB101 live for a similar length of time as those fed on OP50 (Brooks *et al.*, 2009). Indeed, body size was reduced in *C. elegans* fed on *B. s.* MS(+Glu), in comparison to animals fed on *B. s.* MS(-Glu), but the lifespan of these animals was the same. This demonstrates that smaller body size does not entail longer lifespan, and that the two traits can be uncoupled.

#### 4.5.1.2 Egg laying was delayed in *C. elegans* fed on *B. s.* Δ*atp*(+Glu)

Feeding on *B. s.* Δ*atp* affected the amount and/or the pattern of egg laying. In the presence of glucose, egg laying peaked a day later than the other conditions, but was sustained for a longer period after this, indicating a delay in sperm-

depletion. Unfortunately, total brood size in individuals fed on *B. s.* Δ*atp*(+Glu) could not be assessed as a result of escape. However, by day 7 the total brood size was only 50 progeny fewer than animals fed on *B. s.* MS(+Glu), indicating that overall brood size was not dramatically reduced by this diet.

A diet of *B. s.* Δ*atp*(-Glu) induced a significant (15%) reduction in brood size in comparison to animals fed MS(-Glu). This demonstrates that it affected aspects of *C. elegans* physiology even though lifespan was unaffected and indicates that improved health comes at a cost to reproduction in these individuals. *B. s.* Δ*atp*(+Glu) also reduced egg laying, but the effect was even more pronounced. Reducing food levels on solid media beyond a certain threshold typically leads to a proportionate reduction in brood size, reflected in fewer progeny laid on each day (Chen *et al.*, 2009; Tain *et al.*, 2008). The phenomenon observed in this work, where eggs continue to be laid at later ages, has been observed in *C. elegans* undergoing DR. For example, *eat-2* mutants have a similar pattern of an extended reproductive period and higher production of progeny later in life (Huang *et al.*, 2004; Hughes *et al.*, 2007), indicating delayed reproductive ageing. It should be noted that other interventions have produced a similar effect without DR. For example, indoles released from commensal *E. coli* K12 extended the reproductive span of *C. elegans,* possibly by improving oocyte quality or viability (Sonowal *et al.*, 2017), without increasing maximal lifespan. Indoles activated a distinct pattern of gene expression to improve healthspan that was largely unrelated to common longevity pathways. In order to investigate the role of DR even more comprehensively, the level of fat stores - which are reduced upon DR – should be measured in these animals in future. As well as this, dietary restriction in *C. elegans* has been associated with a characteristic shift in autoflourescent age pigments (Gerstbrein *et al.*, 2005). Measurement of this autofluorescence would help to discern whether the physiology of *C. elegans* is in a DR state.

*C. elegans atx-2*, a homolog of mammalian ataxin-2*,* was previously identified as a coordinator of a number of DR-related phenotypes including reduction of development rate, body size, fat levels and brood size, via mTOR signalling (Bar *et al.*, 2016). The physiology of animals fed on *B. s.* Δ*atp*(+Glu) is consistent with a coordinated DR response. *B. s.* Δ*atp*(-Glu) also reduced egg laying, delayed development and increased motility. However, the large size and normal lifespan of animals fed on *B. s.* Δ*atp*(-Glu) indicates that they were not

undergoing DR, although this is complicated by measurements of gene expression (see below). Strikingly, *C. elegans* fed on Δ*atp*(+Glu) were the smallest in size, whilst those fed on Δ*atp*(-Glu) were the largest, whereas fecundity was impaired in both cases, revealing a lack of correlation between body size and fecundity. The largely distinct response of *C. elegans* to *B. s.* Δ*atp*(-Glu) versus *B. s.* Δ*atp*(+Glu) perhaps indicates that different mechanisms underlie reduced fecundity in each case (delayed egg laying in animals fed on *B. s.* Δ*atp*(+Glu) is discussed further below).

### 4.5.2 Was Δ*atp* hard to eat?

#### 4.5.2.1 Pharyngeal pumping was not reduced by *B. s.* Δ*atp*

The rate of pharyngeal pumping on each of the diets supports the argument that *C. elegans* was consuming *B. s.* Δ*atp* efficiently. In the large majority of the population that were found within regions of *B. s.* Δ*atp*(+Glu) bacterial growth, no reduction in the rate of pumping was apparent. It is, however, important to note that a higher number of nematodes were observed to be fully or partially outside of the bacterial food on NGM(+Glu) plates seeded with *B. s.* Δ*atp*. In these animals, pumping was reduced such that the data points were outliers in Fig. 4. 6, although they were observed to return to patches of food and resume normal pumping periodically. This raises the possibility that overall food intake was reduced in these animals by virtue of their increased roaming.

Regardless of pumping rate, certain factors can obstruct feeding. Previously, development of *C. elegans* was found to be slowed in proportion to increasing bacterial cell size (Avery and Shtonda, 2003), and *C. elegans* showed aversion to these same low-quality diets (Shtonda and Avery, 2006). As described, *B. s.* Δ*atp* delayed development with and without glucose, though more severely in its presence. However, a comparison of cell size revealed that, although cells of *B. s.* Δ*atp* were larger in width than the MS, the width of all cells was well within the range of edibility (Avery and Shtonda, 2003). Glucose increased cell length, but there was little difference in length between *B. s.* MS and Δ*atp*. It seems clear, therefore, that differences in cell size did not alter the edibility of the diets. Interestingly, although *B. s.* Δ*atp* could be considered a low-quality diet due to its effects on development, there was no indication that *C. elegans* avoided *B. s.* Δ*atp*(-

Glu). Perhaps, however, a food preference assay in which *C. elegans* is presented with *B. s.* MS or *B. s.* Δ*atp* would reveal reduced preference for this diet.

#### 4.5.2.2 Extracellular matrix formation obstructed feeding

Properties of the bacteria other than cell size could affect edibility (including spores, discussed in Chapter 6). One such factor is the formation of an extra-cellular matrix. Biofilms of *B. subtilis* have characteristic wrinkles that result from the production of a matrix, which is composed of an exopolysaccharide in combination with amyloid-like fibres (Vlamakis *et al.*, 2013). Mutations harboured by *B. subtilis* 168 strains interfere with collective behaviours and result in low biofilm proficiency (Donato *et al.*, 2017), in particular a deficiency in exopolysaccharide (EPS) production (Gallegos-Monterrosa *et al.*, 2016). However, the nutrient profile of the growth medium can have dramatic effects on biofilm structure and development. One study found that the addition of glucose to liquid LB medium enabled a majority of *B. subtilis* 168 variants which were not previously biofilm-forming to form complex, structured colonies (Gallegos-Monterrosa *et al.*, 2016). Moreover, low oxygen levels stimulate the formation of a matrix in *B. subtilis,* presumably as an adaptation to increase surface area for oxygen intake (Kolodkin-Gal *et al.*, 2013). This is mediated by two kinases, KinA and KinB, which respond to impaired electron transport in the mitochondria, or a decrease in the ratio of NAD+ to NADH, leading to higher extra-cellular matrix production and consequent colony wrinkling (Kolodkin-Gal *et al.*, 2013). Although the activity of the MRC appears to be particularly active in *B. subtilis* with mutations in ATP synthase, the level of reducing equivalents is raised (Santana *et al.*, 1994) possibly triggering the biofilm response. Indeed, heightened matrix formation as a consequence of respiration-deficiency, and an exacerbation of this by the addition of glucose, was observed in the work presented here. Increased matrix formation in respiration-deficient *B. subtilis* is also likely to be stimulated by increased secretion of acetic acid (Santana *et al.*, 1994), which acts as a volatile signal to promote the formation of biofilm in *Bacillus* (Y. Chen *et al.*, 2015).

Interestingly, respiration deficiency also controls entry into sporulation (Fig. 4. 14). Low-level activation of the master regulator gene Spo0A by phosphorylation (Spo0A~P) induces matrix formation, whereas a higher level induces sporulation (Mielich-Süss and Lopez, 2015). Impaired oxidative phosphorylation and low $NAD^+$ levels are proposed to act as direct signals for both biofilm formation and

sporulation (Kolodkin-Gal *et al.*, 2013). This can explain why sporulation was seemingly more prevalent in lawns of *B. s.* Δ*atp* (though this was not quantified).

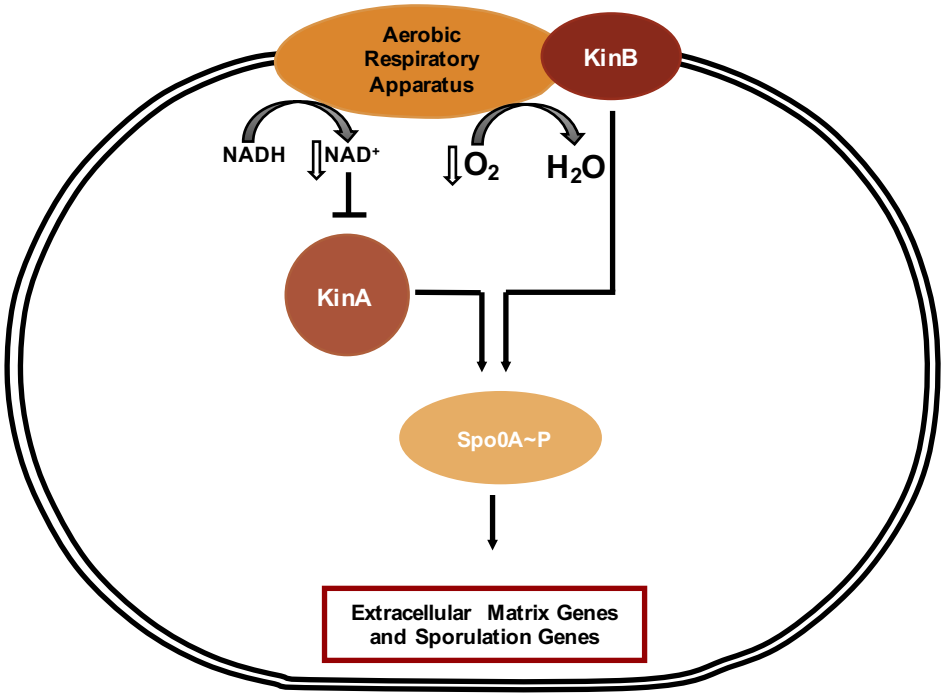



**Figure 4. 14 Model for the control of matrix formation and sporulation by impaired respiration in *B. subtilis*.** See text for details. A similar process is proposed to underlie the enhanced matrix formation seen in lawns of *B. s.* Δ*atp*. Figure reproduced from Kolodkin-Gal *et al.* (2013).

There is considerable evidence that matrix formation, and excessive overgrowth in particular, affected the edibility of *B. s.* Δ*atp*. Firstly, the matrix was hard and very resistant to dissociation. This clumping is likely to have increased the effective cell size, and possibly obstructed feeding as a consequence. Secondly, in situations where nematodes were cultured in lower density, *C. elegans* were aversive to *B. s.* Δ*atp*(+Glu). In particular, during the fecundity assay, in which single individuals were cultured per plate, escape was so pronounced that all 20 nematodes that were introduced onto Δ*atp*(+Glu) had escaped by day 7 of adulthood prior to the conclusion of egg laying. The simple explanation for this is that, owing to the fact that nematode density must be maintained at one worm per plate for these assays, lawn growth and matrix formation was not kept in check to the same extent as during a lifespan experiment (where each plate contained 15-25 nematodes). Additionally, there was less zone 3 growth stimulated by nematode movement over the agar, which proved to be the preferred growth zone. As a result of this, the food was largely inedible for *C. elegans* and escape was prompted.

Similar reasoning can explain the severe developmental delay/L1 arrest and increased roaming that was observed when *C. elegans* were fed from hatching on *B. s.* Δ*atp*(+Glu), as larvae failed to keep bacterial growth in check. The individually-plated animals also had prolonged reproductive periods, which is characteristic of DR. In this case, the delayed fecundity that was seen in *C. elegans* fed on *B. s.* Δ*atp*(+Glu) is likely to have arisen through DR but might not be reflective of the physiology of individuals cultured at higher density (as in lifespan assays). Finally, the lawn location data indicate that *C. elegans* avoided regions of thick matrix formation (primarily in the central lawn) in the presence of glucose. This was more pronounced in the presence of *B. s.* Δ*atp*(+Glu) rather than MS(+Glu). It can be concluded, therefore, that overgrown *B. s.* Δ*atp*(+Glu) represented a low-quality diet because it was hard to eat and was avoided by *C. elegans* as a result.

Was low matrix edibility relevant for the ability of *B. s.* Δ*atp*(+Glu) to increase lifespan and enhance motility? As described, escape behaviour was occasionally high in animals fed on lawns of *B. s.* Δ*atp*(+Glu) during the lifespan experiments, indicating some difficulty with feeding on *B. s.* Δ*atp*(+Glu). Importantly, however, a correlation between censoring as a result of escape and feeding on the ATP synthase mutant was not always observed. For example, when *C. elegans* were fed on *B. s.* MS(+Glu) or Δ*atp*(+Glu) from L1 until death (Chapter 3), essentially the same number of nematodes escaped from plates seeded with either diet (11 and 10 animals, respectively). In this experiment, Δ*atp*(+Glu) dramatically increased lifespan (by 52%). Similarly, the level of escape by N2 *C. elegans* fed on each of these diets was similar during the lifespan experiments presented in the current chapter. Escape was, however, elevated in *eat-2(ad1116)* animals fed on *B. s.* Δ*atp*(+Glu) (Appendix Table B13) compared to those fed on MS(+Glu), perhaps indicating that an inherent difficulty in consuming *B. s.* Δ*atp*(+Glu) was exacerbated by the pharyngeal defect of these mutants. Although the precise relationship between roaming, escape and food shortage is unclear, longer lifespan of *C. elegans* fed on *B. s.* Δ*atp*(+Glu) did not always correlate with higher frequency of escape.

Like escape from the NGM, roaming behaviour across unseeded areas of the plate was often, but not consistently, increased by *B. s.* Δ*atp*(+Glu). However, supporting the notion that avoidance of *B. s.* Δ*atp*(+Glu) was not necessary for longevity, the lawn location data indicate that nematodes only avoided areas of

bacterial overgrowth, and not the bacteria *per se*. Many nematodes remained within less overgrown areas of bacterial growth (zone 3), and relatively few were found outside of any bacteria. These same individuals had considerably extended lifespan (see Fig. 3. 4, Chapter 3). Importantly, zone 3 growth only arose as a consequence of roaming behaviour; however, the extent of and preference for zone 3 growth was similar regardless of whether the NGM(+Glu) was seeded with *B. s.* MS or Δ*atp.* Nematodes fed on MS(+Glu), in which matrix growth was less prevalent, were also often largely within zone 3, showing that thinner regions of growth were preferred regardless of matrix formation. Clearly, however, this analysis only provided a brief 'snapshot' of nematode behaviour on each of the diets. It remains possible, therefore, that roaming was indeed elevated in animals fed on *B. s.* Δ*atp*(+Glu) at other timepoints. In particular, the delay between matrix formation over the central lawn and the growth of potentially more accessible (zone 3) bacteria as a result of nematode movement might be crucial for food availability. In future, it would be beneficial to increase the resolution of the experiment by tracking individuals throughout the entire course of the experiment, using tracking software. Moreover, the construction of *B. s.* MS and Δ*atp* strains that express fluorescent proteins will allow direct monitoring of food intake.

### 4.5.3 The effect of chloramphenicol implicates bacterial overgrowth as causal to longevity

As discussed, the longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu) might have arisen as a consequence of changes in physical lawn properties. Chloramphenicol was included in order to slow bacterial growth and limit the formation of a matrix – in fact, no visible matrix formation was apparent in the presence of chloramphenicol. Δ*atp*(+Glu) was no longer able to extend the lifespan of *C. elegans* under these conditions, supporting the interpretation that matrix growth played an important part in longevity. An alternative explanation for the probiotic effect of *B. s.* Δ*atp*(+Glu) is that a lifespan-extending factor was produced. Chloramphenicol-treated bacteria were still metabolically-active and were in fact able to grow and divide (albeit at a reduced rate). It is possible that a life-extending compound was only able to extend lifespan in high concentrations, necessitating the presence of abundant bacteria. However, if such a metabolite was responsible for longevity induced by *B. s.* Δ*atp*(+Glu), it is surprising that longevity was completely abolished by limiting bacterial proliferation, rather than being unaffected or simply reduced.

FUdR similarly slowed lawn growth and prevented extra-cellular matrix formation (Chapter 3). In this case, however, *B. s.* Δ*atp* was able to extend lifespan in the presence and in the absence of glucose. This would indicate that overgrowth and matrix formation are not important in the longevity of animals fed on *B. s.* Δ*atp*(+Glu). It is important to note, however, that FUdR has broader effects on the bacteria, changing metabolism in such a way as to prevent reproduction in *C. elegans.* FUdR altered bacterial consistency markedly, and hard matrix growth was prevented even when the lawns had become thick. In contrast, the effect of chloramphenicol is likely to be largely restricted to slowing the rate of bacterial growth. The low concentration of chloramphenicol that was used (0.7 μg/ml) ensured that unintended effects on *C. elegans* were unlikely. Much higher concentrations (100 μg/ml) alter lifespan (Houtkooper *et al.*, 2013), and even higher concentrations lead to L3 larval arrest when the chloramphenicol is added on top of the bacterial lawn (Tsang *et al.*, 2001). Notably, the addition of chloramphenicol did not dramatically alter the shape of the mortality curves compared with experiments without this antibiotic, whereas FUdR shifted mean and maximum lifespans considerably (as discussed in Chapter 3). Together, this indicates that chloramphenicol did not, like FUdR, have off-target effects on *C. elegans* lifespan. Nevertheless, in the presence of FUdR, *B. s.* Δ*atp* clearly extended lifespan in a way which did not depend on the formation of an extra-cellular matrix, or even the presence of glucose. Understanding the mechanism behind this could provide valuable insight into why *B. s.* Δ*atp* is beneficial to *C. elegans.*

It remains possible that the production of a specific life-extending metabolite by Δ*atp*(+Glu) was dependent on chloramphenicol-sensitive translation. To investigate this further, it will be useful to measure the lifespan of *C. elegans* fed on *B. s.* MS(+Glu) and Δ*atp*(+Glu) in the presence of a range of other antibiotics. If these similarly abolish longevity whilst affecting matrix formation, then abundant bacterial growth *per se* rather than a change in metabolism is likely to underlie the ability of the *B. s.* Δ*atp*(+Glu) to increase lifespan. In particular, the use of an antibiotic which interferes with the synthesis of the bacterial cell wall – such as carbenicillin – would enable growth to be more specifically targeted.

Fig. 4. 15 summarises ideas linking *C. elegans* lifespan with lawn growth and metabolite production by *B. s.* Δ*atp*(+Glu).

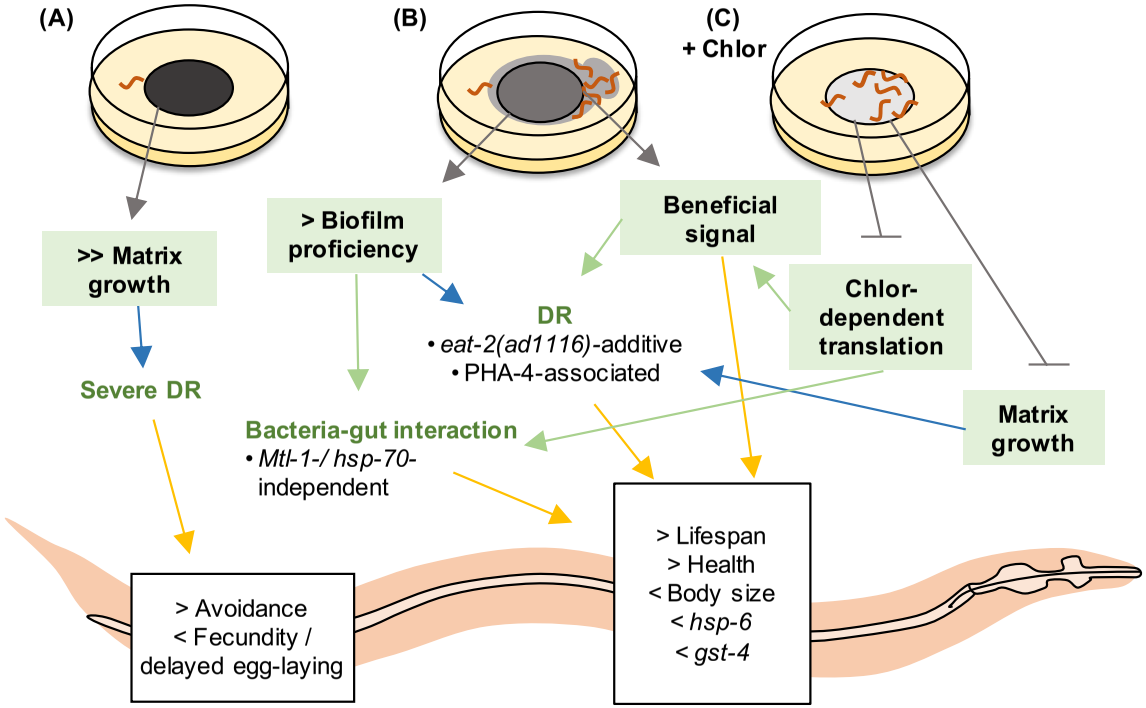



**Figure 4. 15 Possible mechanisms by which *B. s.* Δ*atp*(+Glu) extends the lifespan of *C. elegans*.** Depicted are NGM(+Glu) plates seeded with *B. s.* Δ*atp* that has grown for 48 hours. With low nematode density or introduction of 50-100 L1 larvae (A), extracellular matrix overgrowth reduces bacterial edibility, induces avoidance and L1 arrest and delays reproductive senescence, consistent with DR. With higher nematode density (B), this is largely alleviated. However, the increased biofilm-forming proficiency and matrix formation by Δ*atp*(+Glu) potentially obstructs feeding (milder DR), or promotes longevity through interactions in the intestine (or a combination of the mechanisms). Alternatively, *B. s.* Δ*atp*(+Glu) produces beneficial signals e.g. nitric oxide, fermentation products. (C) Addition of 0.7 µg/ml chloramphenicol (Chlor) abrogates longevity, either by limiting matrix growth and thus increasing lawn edibility, or by preventing the production of beneficial signals that depend on Chlor-sensitive translation. The bacterial activities that are potentially affected by growth of *B. s.* Δ*atp* on 2% glucose plates are shown in green boxes. Blue arrows represent passive mechanisms (reduced food intake, nutritional differences), green arrows reflect active bacterial mechanisms. Yellow arrows represent unknown effectors.

When *B. s.* Δ*atp*(+Glu) is considered, the effect of chloramphenicol addition was two-fold. Firstly, no pro-longevity effect was apparent in the presence of this antibiotic. Secondly, glucose reduced nematode lifespan even below that of animals fed on MS(-Glu). Glucose also reduced the lifespan of *C. elegans* fed on *B. s.* MS. This provides clear evidence that glucose can in principle be toxic to *C. elegans* fed on *B. subtilis,* as studies using *E. coli* suggest. The simplest explanation linking reduced bacterial proliferation to increased glucose toxicity is a reduced rate of glucose removal from the NGM as previously discussed. Another striking observation was that, in the presence of chloramphenicol, a diet of *B. s.* Δ*atp*(-Glu) extended lifespan in comparison to animals fed on Δ*atp*(+Glu) and MS(-Glu). The reason for this is not clear, but it is likely that DR played a role. As described previously, *B. s.* Δ*atp*(-Glu) had visibly impaired growth in comparison to MS. This was compounded by the addition of chloramphenicol, which reduced growth rate even further and might have led to DR as a result of insufficient bacterial growth. Further investigation into this would be warranted, however, as no animals escaped over the course of the experiment, indicating that roaming behaviour was low.

### 4.5.4 The role of DR-associated signalling in the response to *B. s.* Δ*atp*(+Glu)

As discussed, *C. elegans* is likely to have had difficulty feeding on areas of excessive extracellular matrix growth. During most of the experiments presented here, however (including the lifespan and health assays), feeding on *B. s.* Δ*atp*(+Glu) appeared to be largely normal as indicated by normal pharyngeal pumping and dwelling within more accessible bacterial growth. Still, in one view, the longevity of *C. elegans* fed on this diet could simply reflect a milder induction of DR responses as a consequence of limited food intake.

Therefore, the activity of DR pathways in *C. elegans* was investigated. Failure of a particular intervention to further extend the lifespan of long-lived *eat-2* mutants provides good evidence that the intervention induces DR to promote longevity. As the results in this chapter show, however, *eat-2(ad1116)* mutants lived even longer when provided with a diet of *B. s.* Δ*atp*(+Glu), indicating that perhaps DR cannot explain longevity. Importantly, though, additivity of *eat-2* mutation and *B. s.* Δ*atp*(+Glu) does not exclude DR as the sole explanation for *B. s.* Δ*atp*-induced longevity. Whether DR-induced longevity in *eat* mutants is maximised has not been fully established (Mair *et al.*, 2009), so there is potentially room for greater longevity

as a result of DR. The issue is complicated by the finding that, in certain cases, different DR regimes are additive. For example, the lifespan of *eat-2* mutants can be further extended by culture in axenic medium (Houthoofd *et al.*, 2003) and sDR (Greer and Brunet, 2009). As a result, it is conceivable that DR was being elicited by *B. s.* Δ*atp*(+Glu) through pathways that are not activated by mutation of *eat-2.* Moreover, *eat-2* animals undergo constitutive DR from hatching until death, complicating comparison with N2 animals.

In order to better understand whether *C. elegans* was undergoing a physiological response consistent with DR, the expression of genes that have been previously shown to have an essential involvement in various DR regimes was measured using qRT-PCR. Day 2 adults were chosen for analysis of gene expression*; C. elegans* had therefore been fed in the presence of glucose for 48 hrs by the point that the +Glu samples were collected. This timepoint was selected because many of the effects of *B. s.* Δ*atp* were already apparent by this point – including changes in body size, fecundity, stress reporter expression and health. However, any changes in gene expression will only have become apparent relatively recently and including later timepoints would be informative. These data are ambiguous, and any changes were not dramatic in the presence of glucose. However, there is some indication that *C. elegans* fed on *B. s.* Δ*atp*(+Glu) are undergoing DR. The expression of PHA-4 and DAF-9 seems to be increased in animals fed on this diet, though this will need further inquiry. The relationship of these genes to DR is shown in Fig. 4. 16. Regulation of the same pathways was, surprisingly, even clearer in animals fed on Δ*atp*(-Glu) (discussed further below).

Revealingly, there was considerable variability in the recorded lifespan of animals fed on *B. s.* Δ*atp*(+Glu). The mean lifespan increase afforded by Δ*atp*(+Glu) was 17% in one experiment (Fig. 4. 11), and 42% in another (see Chapter 3). Importantly, variation was minimal in experiments conducted by the same experimenter. The variability arose as a consequence of differences in the lifespan of *C. elegans* fed on *B. s.* Δ*atp*(+Glu), as the recorded lifespan of *C. elegans* fed on the other diets was very similar. This makes sense in the context of matrix formation as a potential inducer of DR; slight differences in experimental protocol between experimenters is likely to have altered the extent of Δ*atp*(+Glu) matrix growth prior to the addition of nematodes, altering the availability of food. In this view, at least a

component of the longevity of these animals arose through feeding obstruction as a result of matrix growth.

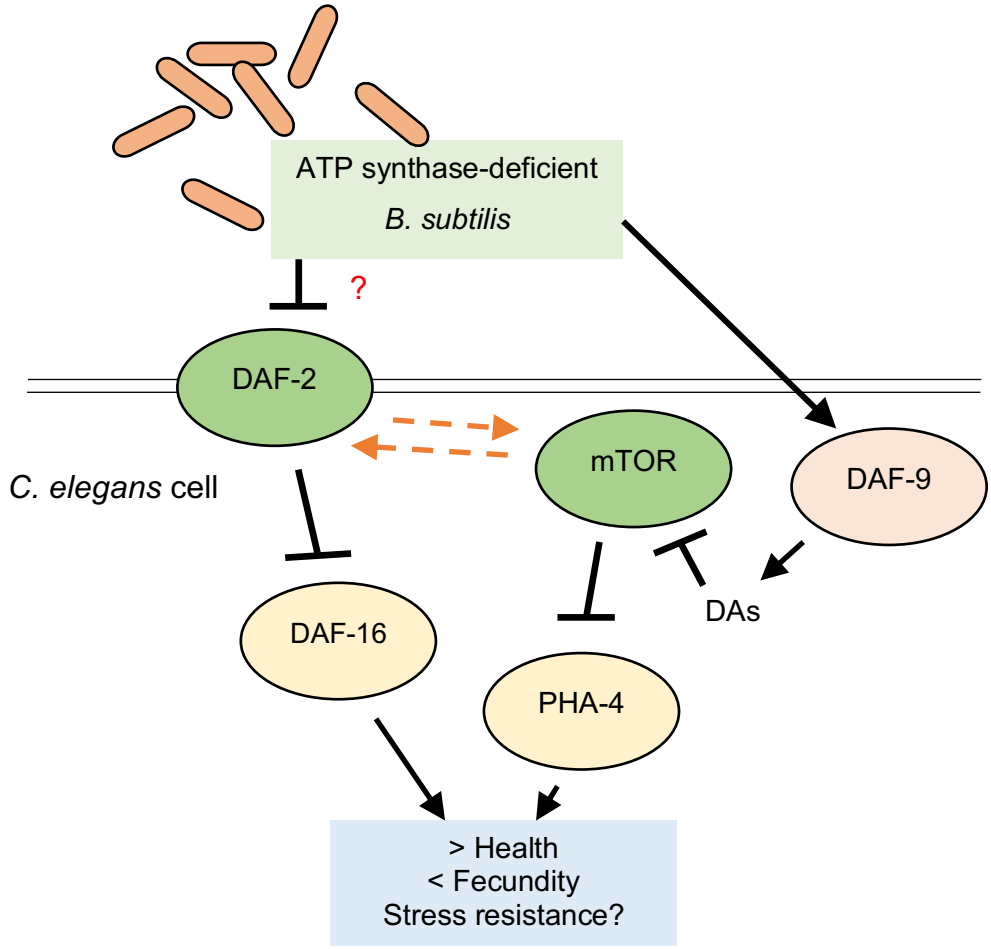



**Figure 4. 16 Signaling pathways that are potentially activated by a diet of *B. subtilis* Δ*atp*.** Possible dietary restriction signaling targets based on qRT-PCR expression data, predominantly without glucose, include steroid signaling (DAF-9)-mediated repression of *let-363*/mTOR. $\Delta^4$-and $\Delta^7$-Dafachronic acids (DAs) produced by DAF-9 inhibit *let-363*/mTOR signaling by binding to the nuclear hormone receptors DAF-12 or NHR8 (Thondamal *et al.*, 2014). Although there is some indication that *daf-16* transcription is elevated by the mutant diet, the involvement of this factor and IIS through the DAF-2 receptor remains to be confirmed. Potential downstream effectors of DR- and IIS-mediated health and stress resistance include increased autophagy and attenuated global translation levels (Hansen *et al.*, 2008). Multiple possible methods of cross-talk exist between mTOR and IIS (orange arrows) (Johnson *et al.*, 2013).

There is some indication that the expression of *daf-16* might be increased by a diet of *B. s. Δatp,* particularly in the absence of glucose, although the expression of *sod-3,* a direct DAF-16 target, was not influenced by diet. It would be useful to increase the statistical power of the test by collecting more biological replicates to

clarify this. Alternatively, the involvement of this pathway could be more definitively assessed by comparing the lifespan of a *daf-16* mutant of *C. elegans* fed on *B. s.* Δ*atp*(+Glu) to that of the wild-type fed on the same diet. Even so, given the broad involvement of IIS signalling in numerous physiological responses, demonstrating that the lifespan extension is *daf-16* dependent will not be sufficient to infer a role of DR.

Revealingly, one study found that the effect of different mutations in IIS signalling, immune response, nutrient sensing and stress response – including *eat-2, hif-1, hsf-1, daf-2* and *daf-16* – on lifespan relative to wild-type *C. elegans* was different depending on whether nematodes were fed on *E. coli* or *B. subtilis* (Sánchez-Blanco *et al.*, 2016). The involvement of many of these pathways in ageing and dietary restriction has not been defined specifically in *C. elegans* fed on *B. subtilis,* and it could be that the set of DR-associated genes that were tested are central only to DR responses in the presence of *E. coli.* There are several other caveats to the interpretation of these data. Firstly, an increase in activity of several of the factors might not be reflected in changes in gene expression. As described, insulin-like signalling through DAF-2 negatively regulates DAF-16 by preventing its translocation into the nucleus, and the activity of SKN-1 is regulated by phosphorylation and O-GlcNAcylation (Li *et al.*, 2017). As a result, quantifying the transcription of genes regulated by these factors is often more informative. As mentioned in Chapter 3, *gst-4* is a transcriptional target of SKN-1, and the lack of *Pgst-4::gfp* upregulation in mutant-fed animals indicates that SKN-1-mediated starvation responses are unlikely to be relevant. To proceed, an unbiased approach such as RNA sequencing would be greatly informative, and in combination with mutants and RNAi would also enable the involvement of non-classical pathways to be elucidated.

### 4.5.5 Does glucose suppress DR?

One of the hypotheses for the beneficial metabolic state induced by DR is an induction of mitochondrial activity to compensate for a temporary deficit in nutritive energy (Ristow and Schmeisser, 2011) – and even reduced glucose in particular (Mobbs *et al.*, 2007). Accordingly, exposing *C. elegans* to a chemical inhibitor of glucose metabolism and glycolysis induces DR by increasing respiration and eliciting mitochondrial ROS-mediated hormesis (Schulz *et al.*, 2007). Whether glucose alone is capable of suppressing DR is unclear, but the antagonistic effect of

glucose and DR on IIS suggests that this might (at least partially) be the case. Clearly, the *eat-2* mutant was capable of undergoing DR, as it had an extended lifespan in comparison to N2 when fed on NGM(+Glu) plates. However, pharynx pumping was impaired in these animals, which will have reduced the intake of glucose from the NGM as well as intake of bacteria. In order to convincingly determine whether *C. elegans* fed on *B. s.* Δ*atp*(+Glu) are undergoing DR, it will be important to clarify 1) whether *C. elegans* are taking up glucose from the NGM(+Glu) when fed on the *B. subtilis* diets, and 2) whether DR pathways can be induced in nematodes taking up glucose, or whether glucose provides an adequate nutrient source. The latter point could be addressed by examining lifespan, fecundity and other phenotypes in *C. elegans* fed in the presence of glucose but in the absence of food.

### 4.5.6 Heightened bacterial biofilm-proficiency is beneficial for *C. elegans*

Interestingly, biofilm-proficient *B. subtilis* strains promote greater lifespan and tolerance to oxidative, heat and pathogen stress in *C. elegans* than biofilm-deficient strains (Donato *et al.*, 2017). These effects are dependent on DAF-16 and HSF-1, but independent of the production of nitric oxide (NO) and competence sporulation stimulating factor (CSF) (Smolentseva *et al.*, 2017). The expression of *mtl-1* and *hsp-70* is required for biofilm-mediated effects, as mentioned. The mechanism by which biofilms promote longevity in *C. elegans* is distinct from DR (Smolentseva *et al.* 2017). Respiration-deficiency and the presence of glucose both lead to enhanced formation of biofilms in *B. subtilis;* therefore, it is possible that a diet of *B. s.* Δ*atp*(+Glu) promoted longevity through a similarly active mechanism related to rudimentary biofilm proficiency and enhanced matrix production.

Importantly, it is not the extracellular matrix *per se* that extends *C. elegans* lifespan and improves thermotolerance, but instead the metabolic state of biofilm-forming *B. subtilis* (Smolentseva *et al.*, 2017). As such, it is still plausible that a higher capacity for matrix formation could underlie the longevity of *C. elegans* even when they resided in regions of the lawn that were not overgrown with matrix structure. An important objection to this interpretation is the observation that metabolically-active *B. s.* Δ*atp*(+Glu) with reduced proliferation were unable to extend lifespan, indicating that the production of metabolites by *B. s.* Δ*atp*(+Glu) is not likely to underlie longevity – unless, that is, the beneficial metabolic pathways

associated with matrix formation by *B. s.* Δ*atp*(+Glu) require chloramphenicol-sensitive translation.

The expression of *mtl-1* and *hsp-70* was measured by qRT-PCR in order to directly test whether such mechanisms are likely to play a role in the longevity of *C. elegans* fed on Δ*atp*(+Glu). There was no indication that the expression of these gene was altered by any of the diets, calling into question the relevance of biofilm-related gene expression for the probiotic effects of *B. s.* Δ*atp.* Research into the biofilm-mediated probiotic effects of bacteria on *C. elegans* is, however, in its infancy, and future studies are likely to uncover novel targets for further investigation. To proceed, matrix formation could be specifically targeted by introducing mutations into the genes of the *tapA-sipW-tasA* and *epsA-G* operons of *B. subtilis*, which encode components of the extracellular biofilm matrix (Vlamakis *et al.*, 2013). Mutations in these genes abolishes the benefits afforded by previously biofilm-proficient strains (Smolentseva *et al.*, 2017). Moreover, gut colonisation is required for the benefits of biofilm in *C. elegans* (Donato *et al.*, 2017; Smolentseva *et al.*, 2017); the expression of the fluorescent protein mKATE under the control of the *tasA* promoter could be utilised to measure intestinal matrix formation.

### 4.5.7 Muscle health was maintained in animals fed on *B. s.* Δ*atp*

Aside from indicating the rate of food intake, the rate of pharyngeal pumping is also a health parameter. Structural and functional declines in the pharynx of *C. elegans* are a feature of ageing in the nematode (Chow *et al.*, 2006). These effects are just one of the manifestations of sarcopenia, or muscle wasting, that occurs as nematodes age (Herndon *et al.*, 2002). The rate of pharyngeal pumping declines rapidly during mid-adulthood, and by day 10 pumping rates are less than one third of their former levels in *C. elegans* fed on *E. coli* (Chow *et al.*, 2006) as a consequence of damage incurred by long-term pumping. Pharyngeal pumping was maintained in animals fed on *B. s.* Δ*atp* even into mid-adulthood. By this timepoint, animals fed on *B. s.* MS were beginning to show a degeneration in pharynx function, and this was particularly true -Glu. It was surprising that pumping rate was consistently lower in animals fed on *B. s.* MS(-Glu), compared to the other diets including *B. s.* MS(+Glu), even in very young adults. Whether *B. s.* MS(-Glu) is detrimental to pharynx function, or whether the presence of glucose is beneficial, remains to be elucidated. Notably, although animals fed on *B. s.* Δ*atp*(+Glu) were

considerably longer-lived, animals fed on Δ*atp*(-Glu) had similar, if not improved, pharynx health. This is consistent with the thrashing assay shown in Chapter 3, which similarly indicated that mutant-induced health benefits were maintained without longevity -Glu.

### 4.5.8 *B. s.* Δ*atp*(-Glu) activated DR pathways in *C. elegans*

Adult expression of *pha-4* appears to be specific to DR-regulated longevity (Panowski *et al.*, 2007), though recently it has been associated with autophagy-dependent longevity upon trehalose accumulation (Seo *et al.*, 2018). Unexpectedly, this gene was markedly upregulated in animals fed on *B. s.* Δ*atp*(-Glu), despite the fact that lifespan was not increased by this diet. Intriguingly, then, this diet can perhaps reveal a novel adult-specific role for PHA-4 activity that is independent from lifespan regulation. As yet, the precise contribution of PHA-4-mediated gene expression to health as opposed to lifespan is unexplored. However, maintenance of autophagy – which is stimulated by PHA-4 – is required for intestinal barrier function and motility in dietary-restricted *C. elegans* (Gelino *et al.*, 2016). Undoubtedly, though, there are other as yet unidentified factors mediated increased health by *B. s.* Δ*atp.* Similarly, *let-363*/TOR seemed to be downregulated (using *act-1,* but not *pmp-3* for normalisation) by this diet and there is some indication that DAF-9 was upregulated. Dafachronic acid production by *daf-9* in *C. elegans* undergoing DR inhibits *let-363/*TOR (Thondamal *et al.*, 2014). However, the involvement of TOR signalling requires further investigation; *bec-1* has been shown to be upregulated in a TOR-signalling dependent manner by DR (Thondamal *et al.*, 2014), but here the expression was unchanged by diet (though it was particularly variable across biological replicates). Investigation into the involvement of steroid signalling in DR again currently concerns lifespan alone. Interestingly, *hsf-1* expression was if anything slightly lower in *B. s.* Δ*atp*(-Glu)-fed animals. Previously, the expression of this gene was upregulated by DR, and knockdown abrogated DR-mediated longevity and stress resistance in different DR regimes (Greer and Brunet, 2009). This indicates that the response of *C. elegans* to Δ*atp* is independent of HSF-1 signalling.

In animals fed on *B. s.* Δ*atp*(-Glu), thrashing was enhanced and pharyngeal pumping was maintained at a high level. Since DR enhances motility and improves muscle health in *C. elegans,* perhaps reduced nutrient intake can account for these phenotypes. However, the body size of *C. elegans* fed on *B. s.* Δ*atp*(-Glu) was not

reduced, and brood size was only slightly lower than that of animals fed on MS(-Glu). Moreover, lifespan was not extended by *B. s.* Δ*atp*(-Glu). DR-induced longevity is often associated with reduced fecundity, but a partial uncoupling of these traits under DR conditions has previously been reported in *Drosophila* (Grandison *et al.*, 2009). However, activation of DR pathways without longevity is a more surprising phenomenon that has little precedent. Indeed, longevity is regarded as a primary hallmark of DR. Possibly, the effect of these signalling pathways on lifespan is counter-acted in these animals by other pathways that remain to be elucidated.

Alternatively, differences in the preparation of the bacteria can explain these findings. As described, concentrated lawns were a more abundant food source that were employed to prevent food shortages, but strikingly activated the $UPR^{mt}$. Unconcentrated lawns were therefore fed to *C. elegans* as indicated, and induced *pha-4* upregulation, enhanced pharyngeal pumping and improved motility. Importantly, as well as *pha-4* upregulation*, C. elegans* fed on unconcentrated Δ*atp*(-Glu) had low expression of *Pacdh-1::gfp,* which is downregulated by starvation (Van Gilst *et al.*, 2005) (Fig. 3. 1, Chapter 3). As growth of Δ*atp*(-Glu) is poor, perhaps the food supply was insufficient, and DR was elicited.

This interpretation is somewhat complicated by the failure of this diet to increase nematode lifespan. Bacterial lawns were unconcentrated from L4 onwards during one of the previous lifespan assays, in which Δ*atp*(-Glu) did not extend lifespan (Fig. 3. 4, Chapter 3). However, the bacteria were concentrated throughout larval development. Could this have prevented Δ*atp*(-Glu) from extending lifespan? This seems unlikely, as DR during adulthood alone is sufficient to increase lifespan; sDR and dietary deprivation regimes can promote longevity in *C. elegans* even when initiated at day 4 or day 8 of adulthood, respectively (Greer and Brunet, 2009; Lee *et al.*, 2006). Indeed, *pha-4* was upregulated in *C. elegans* fed on unconcentrated Δ*atp*(-Glu) at day two of adulthood (Fig. 4. 12), and yet these animals were not long-lived. Body size and fecundity were measured exclusively on concentrated lawns. Notably, whilst animals appeared slightly smaller on unconcentrated Δ*atp*(-Glu) (see Fig. 3. 1), they were large when the bacteria were concentrated (Fig. 4. 2). It will be informative to measure lifespan, motility, body size and fecundity while keeping bacterial preparation fully consistent in order to clarify whether this affects the results.

Further work must therefore be done to determine whether the uncoupling of DR signalling pathways from lifespan, fecundity and body size is simply an artefact of differences in bacterial preparation. If the effect is a biological one, it is interesting to speculate whether a similar phenomenon is true for respiratory-deficient *E. coli* mutant diets. Although these diets increase *C. elegans* lifespan markedly, *E. coli* with coQ-deficiency or ATP synthase mutation do not affect nematode body size or fecundity (Gomez *et al.*, 2012; Saiki *et al.*, 2008). Also, feeding is unaffected by coQ-deficient *E. coli,* and longevity is SKN-1-independent (Gomez *et al.*, 2012), as is the case for *B. s.* Δ*atp*. The activity of DR-related pathways by these *E. coli* diets has not been investigated. It will be worthwhile determining whether such pathways are activated, thus addressing the interesting possibility that respiratory-deficient bacteria act as DR mimetics in *C. elegans*.

### 4.5.9 Induction of DR without reduced food intake

Since poor growth is a property of *B. subtilis* lacking ATP synthase, it is possible that insufficient bacterial growth contributed to DR. Despite this, the rate of pharyngeal pumping of animals fed on *B. s.* Δ*atp*(-Glu) was high, and roaming was minimal. Previous examples of DR induction by bacterial diets – namely, the *Lactobacilli L. salivarius* strain FDB89 (Zhao *et al.*, 2013) and *Weissela* (J. Lee *et al.*, 2015) – have been associated with a reduction in pharyngeal pumping and food intake, as well as increased lifespan, increased motility and reduced body size and fecundity. Interestingly, however, it is possible to elicit a DR-like response in *C. elegans* by activating more downstream pathways, without a reduction in nutrient content or food quantity/uptake. This is desirable, particularly for the end goal of translating research to humans, as the beneficial effects of DR could potentially be achieved without the requirement for a restrictive feeding regimen. Putative DR mimetics include the glycolysis inhibitor 2-DG (Schulz *et al.*, 2007), the polyphenol resveratrol, the chemical NP1 (Lucanic *et al.*, 2016) and the type-2 diabetes drug metformin (Ingram and Roth, 2015). These act on energy metabolism and downstream pathways to induce DR phenotypes in *C. elegans* without reducing food intake (Selman, 2014) (or by methionine restriction in the case of metformin (Cabreiro *et al.*, 2013)).

Fascinatingly, a lack of diffusible microbe-derived signals appears to contribute to the pro-longevity effects of DR by dietary deprivation (Smith *et al.*,

2008). Volatile bacterial cues can similarly shorten the lifespan of *Drosophila* (Libert *et al.*, 2007). Therefore, the effects of DR likely arise from a combination of reduced nutrient intake and reduced food sensing. Moreover, reduced activity of live microbes in the *C. elegans* gut has been implicated in the pro-longevity effect of axenic medium DR (Lenaerts *et al.*, 2008). Here, the extended lifespan of *C. elegans* growth on solid axenic medium was rescued by growth-arrested, metabolically active – but not by dead – bacteria, and was not a consequence of macronutrient restriction or the absence of a diffusible signal from the bacteria. The authors suggest that *C. elegans* requires the presence of live microbes in the gut, possibly for efficient digestion, or perhaps to condition intestinal cells for proper function of pH-dependent nutrient uptake (Lenaerts *et al.*, 2008). Indeed, mutation of oligopeptide transporters in the nematode intestine phenocopies the effects of DR (Nehrke, 2003). Perhaps, then, the impaired respiratory metabolism of *B. s.* Δ*atp* compromises its ability to mediate processes such as these in the nematode intestine, eliciting a DR response.

### 4.5.10 Conclusions and future perspectives

In this chapter, data regarding the involvement of DR in the longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu) are somewhat inconclusive. This diet promotes a lifespan extension which is additive with DR elicited by physical feeding restriction in the *eat-2(ad1116)* mutant and did not seem to impede feeding under the conditions of the experiments. Pharyngeal pumping was normal, and nematodes resided within areas of the lawn that were not overrun by a bacterial matrix. However, when the bacterial matrix overgrew the bacterial lawn, fecundity was reduced and escape behaviour was prominent. The longevity of *C. elegans* fed on *B. s.* Δ*atp*(+Glu) was concurrent with frequently elevated levels of escape and a prominent reduction in body size, as well as a change in the expression of genes which are transcriptionally regulated by DR (although this will need further investigation). Notably, *Pacdh-1::gfp* was downregulated both by starvation and by *B. s.* Δ*atp*(+Glu) (Chapter 3). There is evidence, therefore, that DR contributed to the physiology of *B. s.* Δ*atp*(+Glu)-fed animals, although whether this arose from reduced food intake, or whether distinct, active mechanisms played a part remains uncertain. The data do also seem to point to a mechanism for longevity which is at least partially distinct from DR, and which is sensitive to the effect of chloramphenicol on translation.

Crucially, the activity of the same DR-associated pathways was even more pronounced without glucose, in which nematodes were not long-lived. This calls into question whether such pathways were necessary for the longevity of animals fed on *B. s.* Δ*atp*(+Glu). Instead, perhaps, the regulation of PHA-4 and steroid signalling by *B. s.* Δ*atp* influenced health – indeed, motility was more noticeably affected by Δ*atp*(-Glu) (corroborated and extended in Chapter 6), correlating with higher-magnitude expression changes. It is hoped that the development of RNAi in this system (Chapter 5), or lifespan assays using DR-pathway mutants, can address these questions further.

Both unconcentrated and concentrated lawns of *B. s.* Δ*atp*(-Glu) are potentially suboptimal food sources for *C. elegans.* Moreover, DR through food shortage possibly arises both as a consequence of ATP synthase mutant overgrowth +Glu as well as insufficient growth -Glu. The experiments of this chapter therefore argue for a stricter standardisation of bacterial growth. One promising approach might be to feed *C. elegans* only on spores of *B. s.* MS and Δ*atp.* In this way, physico-chemical properties of *B. s.* MS and Δ*atp*(+Glu) lawns would be very similar, and the confounding effects of matrix formation on food availability could be uncoupled from the effects of live vegetative bacteria inside the gut of *C. elegans.* This approach has been recently used to good effect (Donato *et al.*, 2017), enabling the authors to identify biofilm formation in the gut of *C. elegans* as a major feature of the probiotic effects of a *B. subtilis* diet. Alternatively, liquid culture can also enable food quantity to be carefully controlled, and initial steps have been taken in setting up such a system. Future work, including bacterial metabolomics and RNAseq of *C. elegans*, will enable putative pro-longevity mechanisms to be identified. These will focus on *B. s.* Δ*atp*-responsive gene expression changes in the nematode and the release of glucose-specific metabolites from *B. s.* Δ*atp.* Approaches to distinguishing active from passive explanations for the induction of DR are discussed further in Chapter 6.

# 5 Construction of *B. subtilis* MS and Δ*atp* strains that are capable of eliciting RNA interference in *C. elegans.*

## 5.1 Introduction

### 5.1.1 The principle and use of RNA interference in *C. elegans*

A number of tools exist for probing the genetics of *C. elegans.* One of the most significant of these is RNA interference (RNAi), a simple, specific and efficient tool for characterising loss-of-function phenotypes which was discovered in *C. elegans* (Fire *et al.*, 1998).

The principle of RNAi relies on endogenous pathways in *C. elegans* and other eukaryotic organisms that evolved to respond to nucleic acids derived from pathogenic organisms. The triggers for this response include the presence of exogenous DNA, aberrant transcripts arising from repetitive DNA elements in the endogenous genome, as well as double-stranded RNA (dsRNA) from viral replication. In the classical RNAi pathway, exogenous dsRNA that has entered the cell is processed by the ribonuclease III Dicer into short small interfering RNAs (siRNAs) which degrade homologous mRNAs (Zhuang and Hunter, 2012) (Fig. 5. 1).

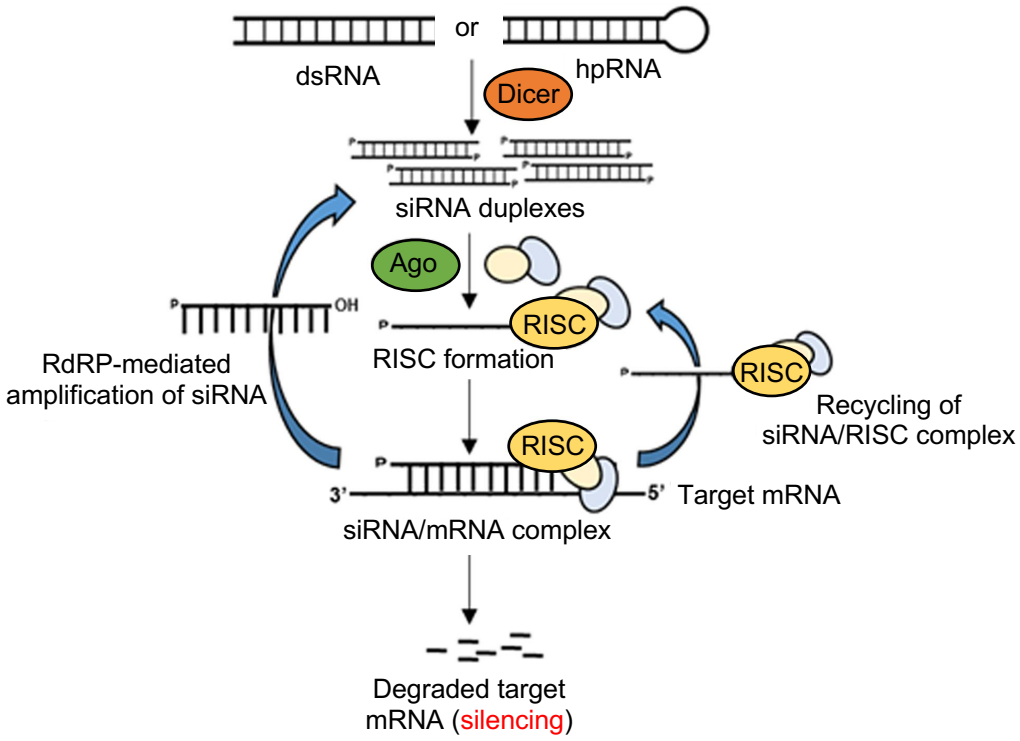



**Figure 5. 1 The classical RNA interference (RNAi) pathway in eukaryotes.** The RNase III enzyme Dicer cleaves long double-stranded RNA (dsRNA) derived from exogenous sources or hairpin RNA (hpRNA) into ~21 nt small interfering RNAs (siRNAs) with a 3' overhang 2 nucleotides in length. A sense passenger strand and an antisense guide strand comprise each siRNA molecule. The passenger strand is degraded, while the guide strand is incorporated into an RNA-induced silencing complex (RISC) to form an siRNA-RISC complex. This complex is guided to highly homologous mRNA molecules by siRNA to initiate endonucleolytic cleavage. Cleavage is carried out by the catalytic component of the RISC complex, induced Argonaut protein (Ago). As a result, translation of the target gene is prevented. Following this, components of the siRNA-RISC complex can participate in generating more siRNAs through the action of RNA-dependent RNA polymerase (RdRP), or they are recycled to form other RISC complexes. Image reproduced from Majumdar *et al.* (2017).

In *C. elegans,* RNAi is particularly potent as a result of ease of dsRNA delivery, and the efficiency of RNA-dependent RNA polymerase (RdRP)-mediated siRNA amplification. As well as this, RNAi in *C. elegans* is systemic in nature; endogenous mechanisms facilitate the spread of silencing signals between cells and tissues (Zhuang and Hunter, 2012). RNAi can be induced by several methods, including microinjection (Fire *et al.*, 1998), soaking in dsRNA (Tabara *et al.*, 1998) or feeding (Timmons and Fire, 1998).

### 5.1.2 RNAi by feeding in *C. elegans*

The least costly and least labour-intensive method to induce RNAi is by feeding *C. elegans* of any stage on dsRNA-expressing bacteria (Timmons and Fire, 1998). RNAi by feeding facilitates rapid and large-scale screening of *C. elegans* for phenotypes of interest. Notably, the effect of gene knockdown can be apparent in the fed individuals, as well as their progeny.

RNAi by feeding relies on the production of dsRNA within the bacterial diet (usually *E. coli*) of *C. elegans.* A feeding RNAi library consisting of over 85% of the *C. elegans* genome cloned into a dsRNA expression vector is commercially available and represents an important resource for nematode biologists. Each *E. coli* strain is capable of inducing a distinct dsRNA and phenotype in *C. elegans.* The original method of RNAi by feeding (Timmons and Fire, 1998) involved cloning a fragment of cDNA from the gene of interest into a feeding vector. The fragment was introduced between two T7 promoters that enabled inducible expression of the dsRNA, and this plasmid was transformed into *E. coli.* Vectors for inducible dsRNA expression require two bi-directional promoters in order to generate both strands of the dsRNA molecule. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is frequently used to induce the expression of dsRNA. The principle of IPTG-inducible gene expression relies on the presence of the lac operon in the expression plasmid. When lactose is absent, the lac repressor protein, LacI, binds to the operator sequence (LacO) and blocks the access of RNA polymerase and gene transcription. Binding of lactose to LacI changes its conformation and prevents its binding to LacO, enabling gene transcription to proceed. IPTG is a molecular mimic of lactose and can therefore stimulate gene transcription by binding to LacI. IPTG is not a component of metabolic pathways, so is not degraded, making it a suitable compound for inducible gene expression (Vavrová *et al.*, 2010). Use of an *E. coli* K12 strain (HT115) lacking *rnc,* encoding double-strand specific RNase III, was shown to improve the efficiency of RNAi (Timmons *et al.*, 2001), and has therefore become the standard diet in RNAi by feeding studies.

Recent studies have proven the principle of using *B. subtilis* as a shuttle organism for dsRNA expression in RNAi experiments (Lezzerini *et al.*, 2015), avoiding the potentially confounding consideration of *E. coli* pathogenicity. Here, the authors designed an expression vector containing two bi-directional IPTG-inducible P*spac* promoters. The system was demonstrated to be as robust as *E. coli*-

mediated RNAi, further expanding the repertoire available to *C. elegans* researchers.

In this chapter, the efficient *B. subtilis* expression plasmid pDR111 is modified to enable the production of dsRNA corresponding to a target sequence. Not only is RNAi by feeding an efficient reverse genetic technique, many ageing-associated *C. elegans* genes including *let-363/*TOR and components of mitochondrial respiration in *C. elegans* (*nuo-2, atp-3*) are lethal or cause developmental arrest when mutated (Rea *et al.*, 2007). With this system, it is hoped that the ageing-associated pathways that mediate the probiotic effects of *B. s.* Δ*atp* can be dissected.

## 5.2 Materials and Methods

### 5.2.1 Reagents

#### 5.2.1.1 Isopropyl-D-thiogalactopyranoside (IPTG)

Prepare a 1M IPTG stock solution by dissolving 238.3 mg of IPTG powder in 1 ml $_{dd}H_20$.

#### 5.2.1.2 *B. subtilis* lysis buffer

Dissolve lysozyme (10 mg/mL) in 30 mM Tris, 10 mM EDTA, pH 6.2.

### 5.2.2 Growth medium

#### 5.2.2.1 LBA containing 1% starch

Make lysogeny broth agar (LBA) as described in Chapter 2. Add 1% (w/v) soluble starch to the mixture and autoclave (20 mins, 121ºC). Pour into 10 cm petri dishes.

#### 5.2.2.2 NGM containing IPTG

Add IPTG to a final concentration of 1 µM or 5 µM into NGM(-Glu).

### 5.2.3 *C. elegans* strains

The wild-type N2 (Bristol) strain was used throughout.

### 5.2.4 *B. subtilis* strains

Strains *B. subtilis* MS Δ*rnc* and *B. s.* Δ*rnc,* ∆*atp* were derived from *B. s.* MS and *B. s.* ∆*atp,* respectively, by deletion of *rncS.* Both strains are therefore spore-forming.

*B. s* MS Δ*rnc* and *B. s.* Δ*rnc,* ∆*atp* were transformed with modified pDR111 (as described below). These strains lack RNase III (see Chapter 2 Table 2. 6).

Transformation of these strains with modified pDR111 resulted in strains *B. s.* ∆*rnc*/pDR and *B. s.* ∆*atp,*∆*rnc*/pDR (as described herein).

### 5.2.5 *B. subtilis* plasmids

In order to induce RNA interference by feeding in *C. elegans,* strains of *B. s.* Δ*rnc* and *B. s.* Δ*rnc,* Δ*atp* expressing double-stranded RNA with homology to a target gene under the control of the LacI-repressible, isopropyl-D thiogalactopyranoside (IPTG)-inducible promoter Phyper-spank were constructed.

The Phyper-spank plasmid pDR111 (Fig. 5. 2) is suitable for expression in *Bacillus* systems (Britton *et al.*, 2002). pDR111 contains a spectinomycin-resistance marker (Spec) as well as a polylinker downstream of the IPTG-inducible Phyper-spank promoter and integrates into the non-essential *amyE* locus of *B. subtilis*. Transformants are therefore rendered resistant to spectinomycin and incapable of starch hydrolysis. The vector was received as a gift from D Rudner, Harvard Medical School (Ben-Yehuda *et al.*, 2003).

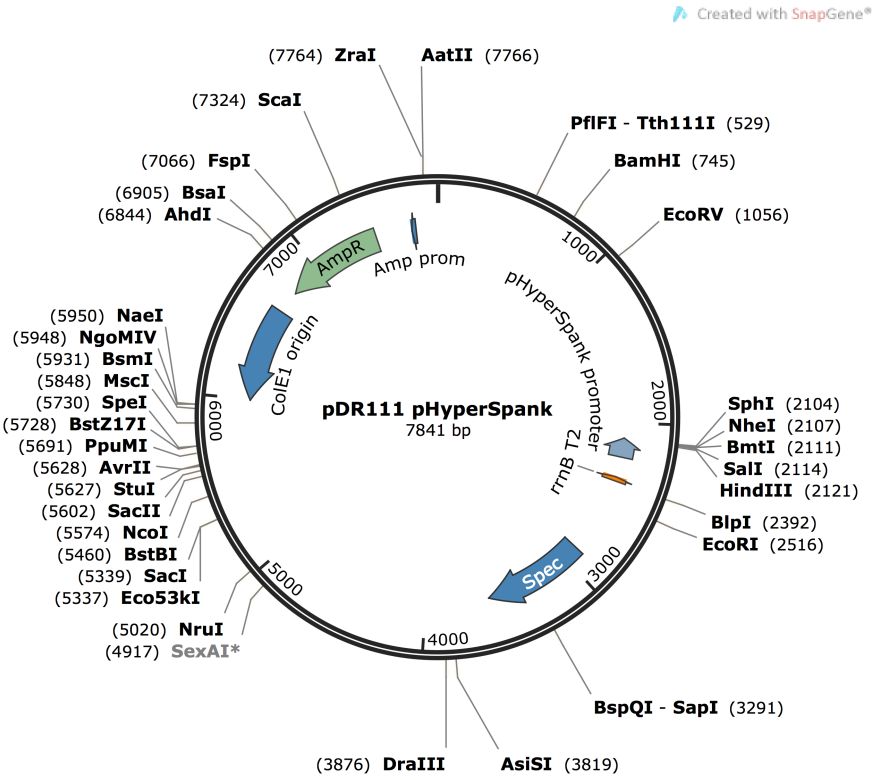



**Figure 5. 2 pDR111 amyE::Phyper-spank (spec) expression vector.** pDR111 is an integration vector for IPTG-induced expression in *B. subtilis.* IPTG-inducible expression occurs through Phyper-spank. In the absence of IPTG, the lac repressor protein, encoded by *lacI*, binds to the operator sequence (LacO) and prevents expression through Phyper-spank. IPTG relieves this inhibition and induces promoter activity (Britton *et al.*, 2002).

### 5.2.6 Oligonucleotides

The primers in Table 5.1 were designed according to certain principles. The length should optimally be 18-22 bp, and the GC content between 40% and 60%, with minimal formation of secondary structures. A primer melting temperature of 55-65 ºC is optimal. Geneious version 10.1.3 was used to design primers (Kearse *et al.*, 2012). Table 5.2 gives primers used for qRT-PCR.

**Table 5. 1 Sequences of primers used in the construction of strains *B. s.* Δ*rnc*/pDR and *B. s.* Δ*atp,*Δ*rnc*/pDR.** Primers 1-3 were used for amplifying the f-*unc-22* and f-Phyper-spank gene fragments. Primers 4 and 5 were used to confirm the presence of two flanking Phyper-spank promoters by Sanger sequencing, following plasmid construction. All primers were designed in Geneious v. 10.1.3.

| # | Primer | Sequence (5'-3') |
|---|---|---|
| 1 | unc_hyper-spank_fwd | ATGATGACCTCGTTTCCACC |
| 2 | unc_rev | TAACAATTAAGCTTAGTCGACAGC |
| 3 | hyper-spank_rev | GTGTTGTTGTCAGTGTTATTGTCG |
| 4 | 195_unc_fwd | GGCCACTTCGTCACAAGGAT |
| 5 | 322_unc_rev | TACCCATCGACCTCCGTCTT |

**Table 5. 2 Primers used in bacterial qRT-PCR analysis.** *sigA* and *recF* were internal controls for expression of *unc-22* in IPTG-induced *B. s.* Δ*rnc*/pDR and *B. s.* Δ*atp*,Δ*rnc*/pDR.

| Target | Primer | Sequence (5’ – 3’) | Source |
|---|---|---|---|
| *sigA* | Fwd. | GTATGCTACGTGGTGGATCAGACAGGCG | E. L. Denham (Warwick Medical School, University of Warwick). |
| | Rev. | CTAATACGACTCACTATAGGGAGACGCAATTTCTTCAGGTGTTGGTTCTCTGC | |
| *recF* | Fwd. | GGTTTCTCCCGTCTACCTTCATGATCTT | |
| | Rev. | CTAATACGACTCACTATAGGGAGAGCATCGTCCGGTCAGTTTGTTTTCTTGT | |
| *unc-22* | Fwd. | Primer # 4, Table 5.1 | This report. |
| | Rev. | Primer # 5, Table 5.1 | |

### 5.2.7 Strategy for inducing RNA interference by feeding

In order to prepare the expression vector for RNA interference, pDR111 required bidirectional Phyper-spank for generation of double-stranded RNA. Therefore, a second Phyper-spank was introduced into the vector.

Two gBlock gene fragments (IDT) were synthesised. Geneious version 10.1.3 (Kearse *et al.*, 2012) was used to design the gene fragments for synthesis. The first (f-*unc-22*) contained a 400 bp sequence taken from cDNA of the *unc-22* gene in *C. elegans* (NCBI database) (blue, Fig. 5. 3A). The characteristic twitching phenotype of *unc-22* nematodes can then be used as a marker for confirmation of RNAi. This fragment was designed to be integrated immediately downstream of Phyper-spank in pDR111 by homologous recombination, and therefore contains sequence homology to pDR111 flanking the *unc-22* sequence. A *Sma*I site (5'-CCC/GGG-3') was introduced at the 5' end of the *unc-22* sequence to enable ligation of the second fragment, f-Phyper-spank. This has sequence homology to the regions flanking the *Sma*I site of f-*unc-22,* enabling the introduction of a second copy of Phyper-spank upstream of the integrated *unc-22* sequence (Fig. 5. 3B).

Both flanking hyper-spank promoters contain downstream *Sph*I sites (5'-GCATG^C -3') in the downstream polylinker. These enable excision of the *unc-22* sequence, and ligation of alternative sequences for targeting genes of interest by RNAi.

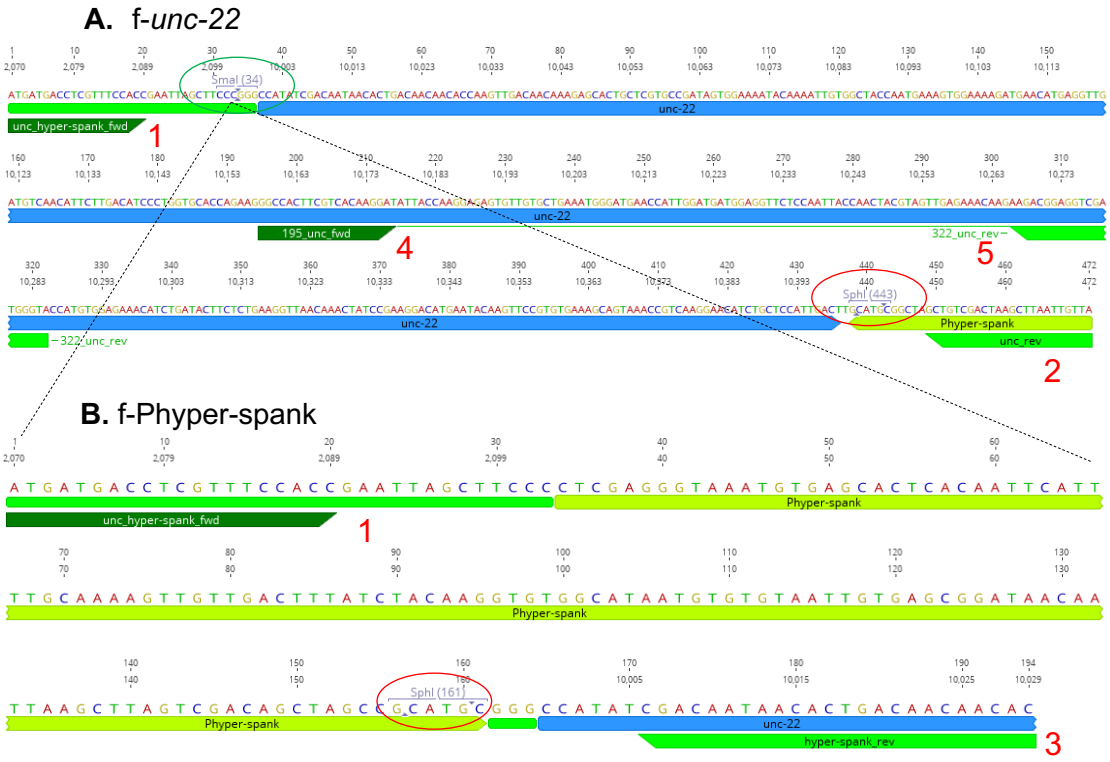



**Figure 5. 3 Design of gene fragments for integration into pDR111.** (A) Fragment f-*unc-22*, containing a 400 bp cDNA sequence from the *C. elegans* gene *unc-22* (blue) flanked by regions of sequence homology to pDR111. Digestion of pDR111 with *Sph*I enables ligation of this fragment downstream of Phyper-spank by Gibson cloning. A *Sma*I site (5'-CCC/GGG-3', green circle) was inserted upstream of the *unc-22* sequence to enable integration of fragment B following digestion. (B) Fragment f-Phyper-spank contains the sequence of a second copy of Phyper-spank (127 bp, pale green) flanked by regions of sequence homology to f-*unc-22.* IPTG-induced expression from the flanking Phyper-spanks will generate *unc-22* dsRNA, resulting in a clear twitching phenotype in *C. elegans*. The *unc-22* sequence can be cloned out, and another sequence cloned in, by digestion with *Sph*I (red circles). Primers (red numbers corresponding to Table 5. 1) are shown beside the gene construct sequences. Geneious version 10.1.3 (Kearse *et al.*, 2012) was used to design the gBlock gene fragments (IDT).

Table 5. 3 gives an overview of the terminology used for constructing plasmid pDR_*unc-22*_hs.

**Table 5. 3 Overview of cloning strategy and terminology.** Linearised plasmid and fragment were ligated to generate the indicated plasmid in each step. The restriction enzyme used to linearise the plasmid in each case is given in brackets.

| Step | Linearised plasmid | Fragment | Plasmid |
|---|---|---|---|
| 1 | pDR111 (*Sph*I) | f-*unc-22* | pDR_*unc-22* |
| 2 | pDR_*unc-22* (*Sma*I) | f-Phyper-spank | pDR_*unc-22*_hs |

### 5.2.8 Molecular biology methods

#### 5.2.8.1 Amplification of gene fragments

To amplify the gene fragments, Phusion® High-Fidelity DNA Polymerase (NEB) was used in a 20 μl reaction volume according to manufacturer's instructions (see Chapter 2 for general methods including PCR).

#### 5.2.8.2 Plasmid isolation from *E. coli*

pDR111 was amplified in *E. coli* by growth at 37 ºC overnight in LB. pDR111 was extracted and purified using the QIAprep Spin Miniprep Kit (QIAGEN) according to manufacturer's instructions. The eluted plasmid was stored at -20 ºC.

#### 5.2.8.3 Assembly of pDR_*unc-22*

Integration of f-*unc-22* into pDR111 to generate pDR_*unc-22* was achieved by Gibson assembly ® (NEB) according to manufacturer's instructions (see Chapter 2, Methods). 1 µl of plasmid and 1 µl of the amplified f-*unc-22* fragment were assembled in a 10 µl final reaction, using 5 µl of Gibson mix. Purified pDR111 was first linearised with *Sph*I according to the suppliers instructions (Promega; Buffer K, 37 degrees). Colony PCR was carried out on positive transformants to confirm the successful integration of f-*unc-22* into pDR111. Primers 1 and 2 in Table 5. 1 were used to amplify the sequence of *unc-22*. The assembled construct was extracted from these colonies and stored at -20 °C.

#### 5.2.8.4 Partial digestion of pDR_*unc-22*

In order to integrate f-Phyper-spank into pDR_*unc-22*, a partial digest of pDR_*unc-22* was carried out to obtain linearised pDR_*unc-22* constructs. This was because the sequence of pDR111 was found to contain a further two *Sma*I sites, along with the introduced site. The partial digest was performed in a 20 µl reaction.

10 µl of pDR_*unc-22* and 0.5 µl of 1/10 diluted *Sma*I were added into the appropriate reaction buffer (10x Buffer J) along with 10x bovine serum albumin (BSA), according to the Promega protocol. The digest was run for 10 minutes at 25 ºC, followed by a 20 minutes heat inactivation at 70 ºC, in a PCR machine. 1 µl of Thermosensitive Alkaline Phosphatase (TSAP) was then added to the reaction in order to dephosphorylate the linearised DNA. The reaction was incubated at 37 ºC for 15 min, followed by 74 ºC for 15 min, as per the Promega protocol.

#### 5.2.8.5 Gel extraction

The optimised *Sma*I partial digest of pDR_*unc-22* was run on a 1% agarose gel, and the band representing the linearised plasmid was identified using a UV transilluminator. A scalpel was used to cut out the relevant band, which was placed inside a 2ml Eppendorf. The QIAquick Gel Extraction Kit (QIAGEN) was used to extract DNA according to the manufacturer's instructions.

#### 5.2.8.6 Assembly of pDR_*unc-22*_hs

Gibson assembly ® (NEB) was again utilised, in order to integrate f-Phyper-spank into *Sma*I-linearised pDR_*unc-22* (1 µl plasmid, 9 µl insert, 20 µl final reaction). Colony PCR using primers unc_hyper-spank_fwd and hyper-spank_rev (1 and 3 in Table 5. 1) was carried out on positive transformants to confirm the successful integration of f-Phyper-spank into pDR_*unc-22*, generating pDR_*unc-22*_hs. The expected product size was 197 bp, corresponding to the sequence of f-Phyper-spank.

#### 5.2.8.7 Confirmation of integration of f-Phyper-spank into pDR_*unc-22*

Following isolation of plasmid DNA from positive transformants, plasmids were digested with *Sph*I-HF (NEB) as follows. 2 µl of plasmid DNA (~ 1 µg) was digested with 0.5 µg of *Sph*I-HF enzyme and 10X CutSmart® buffer for 1 hour at 37 °C, in a total reaction volume of 20 µl. A plasmid control (pDR_*unc-22*) without the second Phyper-spank was also digested. The products of the four reactions were run on a 1% agarose gel. The expected product size was 400 bp, corresponding to the sequence of *unc-22.* As a further check that pDR_*unc-22*_hs had been constructed, plasmids were subjected to Sanger sequencing (Appendix Fig. C3). 5 µl of plasmid DNA to be sequenced and 5 µl of 5 µM primer were sent for Sanger sequencing (GATC Biotech). Primer unc_hyper-spank_fwd confirmed integration of

f-*unc-22*, whilst 322_unc_rev was used to confirm integration of the second Phyper-spank.

#### 5.2.8.8 Transformation of *B. subtilis* with pDR_*unc-22*_hs

Following confirmation that the second Phyper-spank was integrated into pDR_*unc-22*, the resulting pDR_*unc-22*_hs plasmid was linearised with *Sac*I to enable transformation of *B. subtilis*. 2 µl of pDR_*unc-22*_hs was added to 2 µl of BSA (10x) and 2 µl of MULTI-CORE™ buffer (10x) (Promega). 1µl of *Sac*I was added, and $_{dd}H_20$ was added to a final volume of 20 µl. The reaction was allowed to proceed at 37 ºC for 1 hr, at which point the temperature was increased to 70 ºC in order to inactivate *Sac*I. The linearised plasmid was stored at - 20ºC. The *B. subtilis* gene *rncS* encodes RNase III (Wang and Bechhofer, 1997), which would otherwise degrade dsRNA. Colonies of *B. s.* MS lacking RNase III (strain *B. s.* Δ*rnc*), as well as *B. s.* Δ*rnc* lacking *atpA-I* (*B. s* Δ*rnc,* Δ*atp*) were grown on LB agar at 37 ºC. These were then picked into Paris Medium and transformed with linearised pDR_*unc-22*_hs as described in Chapter 2, Methods. LBA containing 100 µg/ml spectinomycin were used to select for positive transformants.

#### 5.2.8.9 Starch hydrolysis test

pDR111 integrates into the *amyE* locus of *B. subtilis,* rendering the bacteria incapable of starch hydrolysis. Transformation of *B. s.* Δ*rnc* and *B. s* Δ*rnc,* Δ*atp* with pDR_*unc-22*_hs was therefore confirmed by a starch hydrolysis test. 16 transformed colonies were picked from 100 µg/ml spec plates and streaked onto a starch plate and a plate lacking starch. Plates onto which *B. s* Δ*rnc,* Δ*atp* was streaked contained 5 µg/ml phleomycin. A control non-transformed colony was included for both strains. Plates were incubated at 37 °C overnight. Iodine, which turns from yellow-brown to blue-black in the presence of starch, was applied to the surface of the agar. After 5 minutes, the presence of a 'halo' around bacterial growth, corresponding to starch breakdown, indicated negative transformants.

#### 5.2.8.10 Collection of IPTG-induced *B. s.* Δ*rnc/*pDR and *B. s.* Δ*rnc,*Δ*atp/*pDR samples for qRT-PCR

Colonies of *B. s.* Δ*rnc/*pDR and *B. s.* Δ*rnc,*Δ*atp/*pDR were inoculated into LB and grown overnight at 37 °C, 200 rpm for 17 hrs. NGM(-Glu) plates containing IPTG at two concentrations (1 µM and 3 µM), as well as control NGM(-Glu) plates lacking IPTG, were seeded with 300 µl of each culture. Two separate cultures of

each strain were set up and seeded onto the plates (two biological replicates). Plates were allowed to grow for 48 hrs at 20 °C. At this point, sterile M9 was added to each plate and a sterile plastic scraper was used to remove the bacterial lawn (three plates per replicate for *B. s.* Δ*rnc*, and ten plates per replicate for *B. s.* Δ*rnc,* Δ*atp* due to poorer growth). Resuspended lawns were centrifuged at 5000 rpm for 8 minutes, and the supernatant was removed. Pellets were flash frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

#### 5.2.8.11 Extraction of RNA from *B. subtilis* Δ*rnc* and Δ*rnc,* Δ*atp*

Bacterial pellets were allowed to defrost and were resuspended in lysis buffer containing lysozyme (10 mg/ml). Samples were transferred to RNase-free 2 ml Eppendorfs and incubated at 37 °C for 30 min to allow the bacteria to lyse. Subsequently, 1 ml of TRIzol (Invitrogen) containing phenol was added to each pellet, as well as 0.3 ml chloroform. RNA extraction then proceeded as described in Chapter 4, Methods. RNA purity was confirmed by running the samples on a 1% agarose gel.

Samples were used for qRT-PCR following cDNA synthesis as described in Chapter 2, Methods.

## 5.3 Results

### 5.3.1 Construction of pDR_*unc-22*_hs

Following digestion of pDR111 with *Sph*I as described in Methods, f-*unc-22* was successfully cloned in to generate pDR_*unc-22.* This was confirmed by PCR using primers 1 and 2 (Table 5. 1), with an expected band size of ~470 bp.

In order to clone f-Phyper-spank into pDR_*unc-22*, a partial digestion of the plasmid was undertaken due to the presence of multiple unexpected *Sma*I sites. The gel in Appendix Fig. C1 shows the result of the partial digestion to obtain linearized pDR_*unc-22* plasmid. A 1/10 dilution of *Sma*I in Buffer J resulted in a high concentration of linearized plasmid at around 8 kb. Following excision of the relevant band from the optimized digestion protocol, the *Sma*I-linearised pDR_*unc-22* fragment was extracted and purified. Gibson assembly of pDR_*unc-22* and f-Phyper-spank was followed by transformation of competent *E. coli* and single colony PCR on positive transformants as described in general methods, Chapter 2 section 2.2. Primers 1 and 3 in Table 5.1 were used (Appendix Fig. C2).

As further confirmation that f-Phyper-spank had been successfully integrated into pDR_*unc-22*, the putative pDR_*unc-22*_hs construct was digested with *Sph*I in order to excise the sequence of *unc-22.* The resulting DNA fragments were run on a gel (Fig. 5. 4). Lanes 1-3, corresponding to digested plasmid DNA from positive Gibson-cloned transformants, drop out the 400 bp *unc-22* sequence. This is absent from lane 4, the negative control (pDR_*unc-22*). Finally, Sanger sequencing using primers 4 and 5 within the *unc-22* sequence (Table 5.1) confirmed the presence of *unc22* now bordered by two Phyper-spank (Appendix Fig. C3).

*B. s.* Δ*rnc* and *B. s.* Δ*rnc,* Δ*atp* were transformed with pDR_*unc-22*_hs. Uptake of the pDR111 plasmid into the *amyE* locus was confirmed using the starch hydrolysis test.

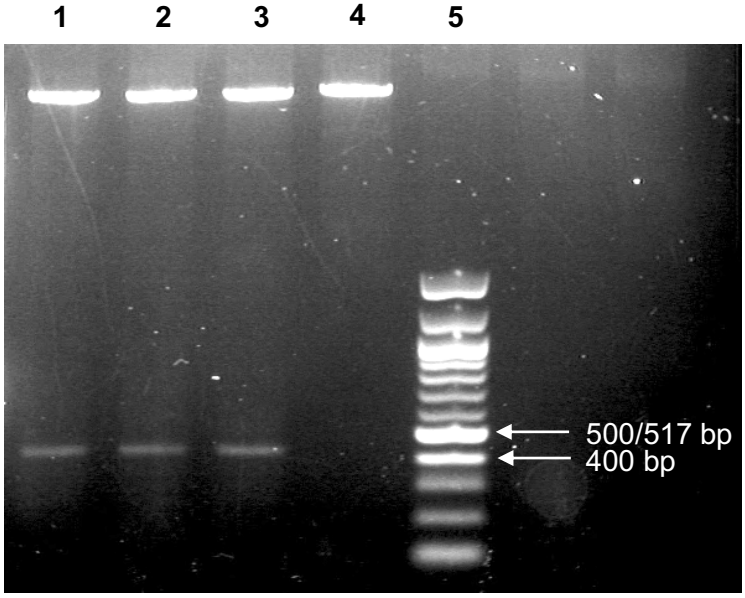



**Figure 5. 4 Drop out of the *unc-22* sequence by *Sph*I digestion of pDR_*unc-22*_hs.** Lanes 1-3, *Sph*I-digested Gibson assembled pDR_*unc-22*_hs transformants; Lane 4, *Sph*I-digested negative control pDR_*unc-22* (without the second Phyper-spank); Lane 5, Quick-Load® Purple 100 bp DNA Ladder (NEB). Lanes 1-3 drop out the expected band size of ~400 bp (the *unc-22* sequence).

### 5.3.2 *C. elegans* fed on *B. s.* Δ*rnc*/pDR and *B. s.* Δ*atp*,Δ*rnc*/pDR did not display the *unc-22* phenotype

NGM(-Glu) plates were seeded with *B. s.* Δ*rnc/*pDR and *B. s.* Δ*atp,*Δ*rnc*/pDR containing IPTG-inducible pDR_*unc-22*_hs. As per the standard methodology, synchronised *C. elegans* were introduced onto these plates and left to become egg laying adults. These were then periodically transferred onto freshly seeded NGM plates containing IPTG every 48 hrs until the end of egg laying and were screened for the twitching phenotype. The progeny of these individuals were also screened. However, the characteristic twitching phenotype of individuals in which *unc-22* has been knocked down was not present.

### 5.3.3 *unc-22* transcription was elevated in IPTG-treated *B. s.* MS Δ*rnc*/pDR and Δ*atp*,Δ*rnc*/pDR

As *C. elegans* were not displaying an *unc-22* phenotype when fed on *B. s.* MS Δ*rnc/*pDR or Δ*atp,*Δ*rnc*/pDR, the expression of *unc-22* was investigated in these strains following growth on IPTG. qRT-PCR analysis of *unc-22* transcript abundance was conducted on *B. s.* Δ*rnc*/pDR and *B. s.* Δ*atp,*Δ*rnc/*pDR grown on

NGM(-Glu) plates with 1 μM or 3 μM of IPTG for 48 hours. Control samples grown on NGM(-Glu) lacking IPTG were also included.

Fig. 5. 5 shows the relative transcript abundance of *unc-22* using *sigA* (A) or *recF* (B) as the internal control. Two independent biological replicates were collected for each IPTG concentration and control samples, and both replicates are plotted. Descriptive statistics and pairwise analysis is given in Appendix Table C1 and C2.

Using *sigA* as an internal control, the abundance of *unc-22* was on average 1000-fold higher in *B. s.* Δ*rnc/*pDR treated with 1 μM IPTG in two independent replicates (ΔCt = -3.7, -3.31666) in comparison to un-induced control samples (ΔCt = 6.947, 6.07) ($p$ = 0.0111, two-sample t-test). The expression of *unc-22* in *B. s.* Δ*atp,*Δ*rnc*/pDR induced with 1 μM IPTG (ΔCt = -1.517, -2.157) was also higher than in controls (ΔCt = 5.277, 8.123), representing a ~250-fold increase in relative expression, although this did not reach statistical significance ($p$ > 0.05, two-sample t-test). The same pattern was true when 3 μM IPTG was used, although *unc-22* expression was if anything slightly lower than in cultures induced with 1 μM IPTG (~700-fold expression increase in *B. s.* Δ*rnc/*pDR, 115-fold expression increase in *B. s.* Δ*atp,*Δ*rnc*/pDR).

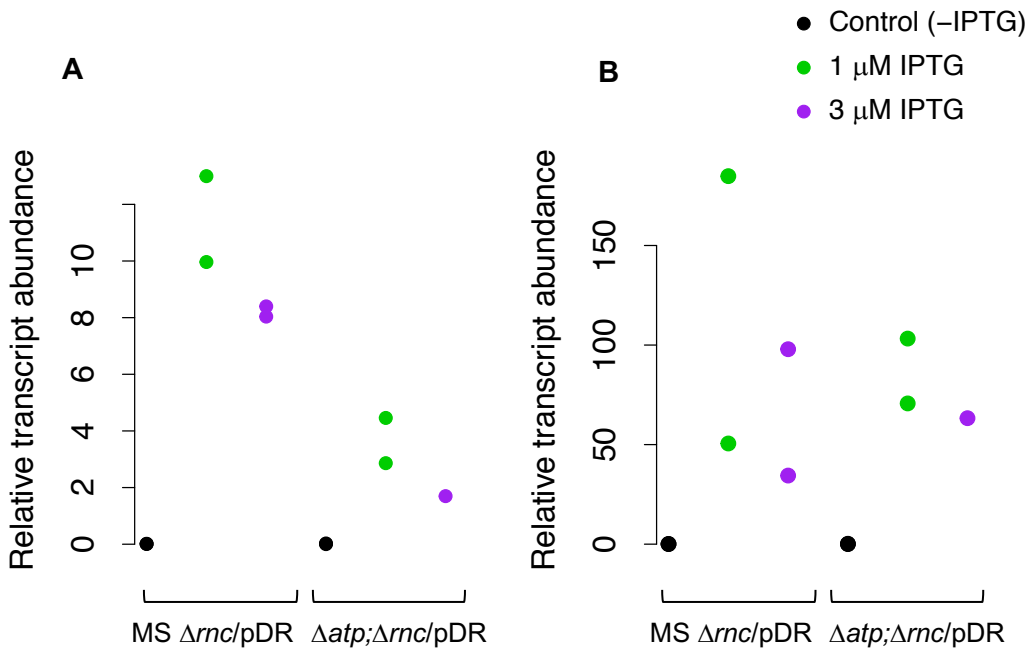



**Figure 5. 5 Expression of *unc-22* in IPTG-induced *B. subtilis* MS Δ*rnc*/pDR and Δ*atp*,Δ*rnc*/pDR in the absence of glucose.** Relative transcript abundance compared to *sigA* (A) and *recF* (B) internal controls was higher in samples that were grown on NGM(-Glu) containing IPTG (1 μM or 3 μM). Two independent biological replicates are shown for each condition (except for Δ*atp*;Δ*rnc*/pDR 3 μM IPTG where the second replicate was excluded from analysis due to very high relative expression values). Primer pair 4 and 5 (Table 5.1) were used to amplify a region of the *unc-22* gene.

### 5.4 Discussion and future directions

The technique of RNA interference by feeding enables rapid, highly specific and large-scale knockdown of target genes. Development of RNAi-competent *B. subtilis* master strain and ATP synthase mutant would enable the pathways that mediate diet-dependent effects in *C. elegans* to be interrogated. It is not clear what accounts for the failure of IPTG-induced *B. s.* Δ*rnc*/pDR and *B. s.* Δ*rnc*,Δ*atp*/pDR to knock down *unc-22* expression in *C. elegans.* Two concentrations of IPTG – 1 mM and 3 mM – were tested. A previous report determined that growth of bacteria overnight without IPTG induction, and subsequently inducing bacteria with IPTG in plates at room temperature overnight, was the most efficient mode of bacterial induction (Kamath *et al.*, 2001). This was the method used here. 1 mM IPTG was also determined to be the optimal concentration for inducing RNAi by feeding in *C. elegans* (Kamath *et al.*, 2001). As suggested, *C. elegans* were allowed to feed on the induced bacteria for a long period of time – up to a week. However, neither 1 mM or 3 mM IPTG was able to induce *B. subtilis* and generate the target phenotype. In fact, 3 mM IPTG was less able to induce the expression of dsRNA, possibly reflecting over-induction of the construct and bacterial lethality (Kamath *et al.*, 2001).

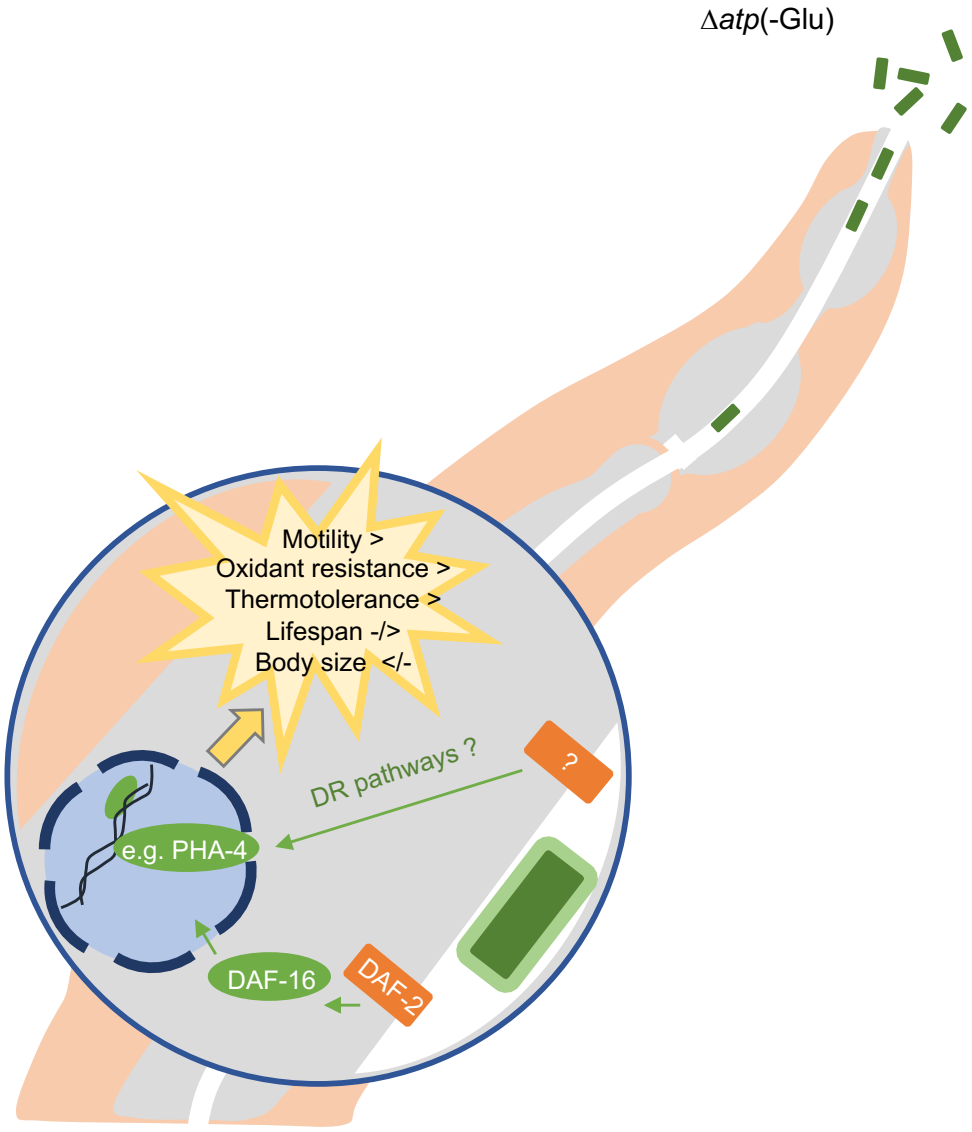
The qRT-PCR results suggest that *unc-22* is expressed from pDR_*unc-22*_hs in both transformed strains in the presence of IPTG. However, it is possible that the level of expression is insufficient to knock down the target gene by feeding in *C. elegans.* This could be more easily addressed by quantifying the concentration of dsRNA rather than using relative expression, and comparing it to the amount produced by IPTG-induced *E. coli* HT115 that is able to elicit the target phenotype in *C. elegans* (~1000 ng/ml bacterial culture for a 700 bp insert) (Xiao *et al.*, 2015). A partial reduction in *unc-22* function can elicit a weak twitching phenotype that is only apparent in the presence of levasimole (Timmons *et al.*, 2001). It will therefore be informative to feed *C. elegans* on the IPTG induced bacteria in the presence of this chemical. The cDNA sequence of *unc-22* that was introduced into pDR111 was selected from the Reference mRNA NCBI database. Although *unc-22* is abundant, with several thousand *unc-22* mRNA molecules in each striated muscle cell (Fire *et al.*, 1998), the sensitivity of particular genes to different methods of RNAi can vary. It might be that substituting in the sequence of a different gene will produce a visible phenotype. Alternative positive control phenotypes include larval arrest, egg laying

defective, dumpy and slow growth mutants, and a future step will be to make use of one of these visible markers. In particular, the use of GFP presents an easily screened visible marker for the efficacy of RNAi. Another possibility is that *unc-22* was only expressed from a single promoter. To address this, dsRNA could be selected for in the total RNA extract using RNase A to degrade single-stranded material. This would also facilitate absolute quantification of dsRNA. Importantly, the Ct values for the internal controls *sigA* and *recF* were similar in both strains (Appendix Table C1), indicating that their expression was largely independent of differences in growth rate. However, *unc-22* expression in IPTG-treated *B. s.* Δ*rnc*,Δ*atp*/pDR was in general lower than in IPTG-treated *B. s.* Δ*rnc*/pDR. Deletion of ATP synthase seems therefore to partially compromise the ability of the mutant to express *unc-22* dsRNA effectively. Although the reason for this is not clear, the Ct values only reflect steady-state transcript production in the bacteria, and differences in transcript turnover might underlie this discrepancy. If the construct still fails to knock down *C. elegans* target genes using different phenotypic markers, it will be worth investigating alternatives to the IPTG-inducible promoter used here (Yang *et al.*, 2017).

Once RNAi is working efficiently in this system, candidate ageing-associated pathways could be targeted in order to investigate the necessity of these components for the enhanced health and/or longevity elicited by *B. s.* Δ*atp*. These include DR-related components that are regulated by a diet of *B. s.* Δ*atp*(-Glu), such as *pha-4, let-363* and *daf-9,* as well as components of IIS and canonical ageing genes. Given the aforementioned possibility of differential food intake on each of the diets, such analysis should ideally be conducted using growth-arrested bacterial lawns of controlled density and cell number. These ideas will be elaborated in the following chapter. During the process of constructing these RNAi-competent strains, the decision was made to shift to spore-less *B. subtilis* 168 strains for further research into ATP synthase-deficient *B. subtilis,* due to the relative difficulty of bacterial transformation in *B. s.* MS and *B. s.* Δ*atp* (Chapter 6). In these spore-less strains, the toxin/antitoxin system makes the *rnc* gene essential; the prophages Skin and SPβ, which are absent from *B. s.* MS and Δ*atp* but present in the spore-less strains, encode two toxins that induce lethality in the absence of RNase III (Durand *et al.*, 2012). Given the benefits of the spore-less strains (discussed in Chapter 6), it will be worth deleting these prophages and subsequently *rnc* so that plasmid pDR_*unc-22*_hs can be introduced.

## 6 Development of *B. subtilis* strain 168 for investigation into the probiotic effects of bacterial ATP synthase deletion

### 6.1 Graphical abstract



## 6.2 Introduction

### 6.2.1 Aims and objectives

Prior investigation into ATP synthase-deficient *B. subtilis*, detailed in Chapters 3 and 4, had established this mutant as an interesting case study for understanding host-microbiota interactions in *C. elegans*. However, progress was impeded by technical challenges. An important requirement for a bacterial system is relative ease of genetic manipulation. Although *B. subtilis* excels in this regard, the particular *B. s.* strain utilised in the previous chapters - *B. s.* MS and the ATP synthase mutant derived from this – proved to be very challenging to transform. In this chapter, the ATP synthase operon was deleted in the parental background of the *B. subtilis* 168 strain utilised previously. This strain was made incapable of spore formation and was facile to genetically manipulate. The lifespan, health and stress response of *C. elegans* on this diet was characterised to direct future work with this strain.

The aims of the experiments in this chapter were to:

- Develop *B. subtilis* strain 168 *trpc2* as a system for investigating the effect of bacterial ATP synthase deletion on *C. elegans* physiology
- Determine the lifespan of *C. elegans* fed on these strains
- Determine the health and stress resistance of *C. elegans* fed on these strains

## 6.3 Materials and Methods

### 6.3.1 Reagents

#### 6.3.1.1 Resuspension solution

10 mM EDTA, 25 mM Tris and 50 mM glucose in $_{dd}H_20$. Store the solution at 4 °C.

#### 6.3.1.2 Paraquat solution

Prepare 1 M paraquat solution by dissolving 0.257 g of paraquat powder in 1 ml $_{dd}H_20$. Store at 4 °C.

### 6.3.2 Growth media

#### 6.3.2.1 Difco Sporulation Media (DSM)

Prepare to the following recipe: 8 g/L bacto nutrient broth, 0.1% (w/v) KCl, 0.012% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001 M NaOH in $_{dd}H20$. Adjust the volume to 1 L with $_{dd}H20$, and the pH to 7.6. Autoclave and allow to cool to 50°C. Add the following solutions prior to use: 0.001 M Ca(NO3)2, 0.1 mM MnCl2, 0.001 mM FeSO4.

#### 6.3.2.2 Nematode growth medium

NGM(-Glu) and NGM(+Glu) plates were prepared as described in Chapter 2.2.

### 6.3.3 *C. elegans* strains

The wild-type N2 (Bristol) *C. elegans* strain was used throughout.

### 6.3.4 *B. subtilis* strains and oligonucleotides

The *B. subtilis* parental strain 168 *trpc2* was selected for deletion of *spoIIE* and *atpA-I.* This resulted in spore-less strains *B. s.* 168 MS and 168 ∆*atp* (see Chapter 2.1 for strain descriptions) which were used throughout as indicated. Table 6. 1 shows the primers used to confirm deletion of *atpA-I* in *B. s.* 168 MS.

**Table 6. 1 Sequences of primers to confirm deletion of *atpA-I* in *B. subtilis* 168 *trpc2*.** Primer 1 binds within the antibiotic resistance cassette that is only present upon generation of the *atpA-I* deletion. Primers 1 and 2 generate a band at 200 bp that confirms the deletion.

| Primer | Sequence (5' - 3') | Source |
|---|---|---|
| 1. Tradis_F | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATCTTCATCGACCTGCACCG | E. L. Denham (Warwick Medical School, University of Warwick). |
| 2. pRMC_ATPase_Rw | AAAAATCCTTCTCATAAAGAGAAG | |

### 6.3.5 Generation of spore-less strains *B. s.* 168 MS and 168 Δ*atp*

The *B. subtilis* transformation methodology described in Chapter 2.2 was used for deleting *spoIIE* (Feucht *et al.*, 1996) in the parental strain by homologous recombination, as well as to delete *atpA-I* (Tanaka *et al.*, 2013) in this spore-less strain.

#### 6.3.5.1 Visual confirmation of spore knockout following deletion of *spoIIE*

To screen efficiently for the presence of spores, three positive colonies of transformed *B. s.* 168 were picked and used to inoculate 5 mls Difco Sporulation Medium (DSM). These were grown overnight at 37 ºC, shaking at 200 rpm, for 24 hours. At this point, glass slides were prepared with 3% agarose pads, onto which 5 µl of each DSM culture was pipetted and covered with a coverslip. The cells were then screened for the presence of phase-bright spores.

If this screen gave a positive result (no spores), the cultures were screened again using the Sterlini-Madelstam sporulation protocol (Sterlini and Mandelstam, 1969).

#### 6.3.5.2 Genomic DNA extraction to confirm deletion of *atpA-I*

Following transformation of spore-less 168 MS with DNA to delete *atpA-I* (Tanaka *et al.*, 2013), 9 positive colonies that grew on phleomycin 5 µg/ml were selected for DNA extraction. Overnight cultures were grown in 5 ml LB medium. 2 ml of each sample was transferred into an Eppendorf tube and centrifuged for 2 min at 14,000 rpm. Supernatant was discarded and 0.5 ml of Resuspension solution

was added as well as 5 mg/ml lysozyme. Samples were incubated for 10 min at 37°C. Then, 20µl (20 mg/ml) RNase, 20µl (20 mg/ml) Proteinase K (Thermo Fisher Scientific), and 25µl 10% SDS were added, and samples were incubated for 10 min at 37°C and 45' at 60°C. 1 volume of Phenol/Chloroform/isoamyl alcohol (25:24:1, v/v) was added and samples were vortexed and centrifuged (10min, 14,000 rpm). The upper phase was transferred to a clean vial, and the procedure was repeated. 1/10 volume of 3M NaAc (pH 5.2) and 1 volume of 96% EtOH was added to the samples, which were then inverted until the DNA condensed. Samples were centrifuged for 5 min at 14,000 rpm, and the supernatant was discarded. Pellets were rinsed with 70% EtOH, dried and dissolved in 500µl TE buffer. Samples were stored at -20 °C until use.

#### 6.3.5.3 Confirmation of deletion of *atpA-I*

The extracted DNA (see above) was used as a template in a PCR reaction to confirm deletion of *atpA-I.* Control reactions were set up with DNA from parental *B. s.* 168 as well as two positive control Δ*atp* templates, and a negative control lacking any template DNA. 2 µl of template DNA and 1 µl of each primer 1 and 2 (Table 6. 1) were added to 10 µl of GoTaq Green Master Mix (GMM, Promega) and $_{dd}H_20$ to a final reaction volume of 20 µl. The PCR settings were as described in Chapter 2. 2, with an annealing temperature of 55 °C. Reactions were run on a 1% agarose gel.

### 6.3.6 Seeding NGM

#### 6.3.6.1 Seeding NGM with *B. s.* 168 MS and 168 Δ*atp*

Seeding of plates was essentially the same as in Chapter 3. To minimise variability in lawn growth, the $OD_{600}$ of overnight cultures of *B. s.* 168 MS and 168 Δ*atp* was measured and standardised to 8 prior to seeding by the addition of a suitable volume of sterile LB (approximately equivalent cell number). Bacteria were seeded onto NGM(+Glu) by diluting in $_{dd}H_20$, using the same methodology as described in Chapter 3.2.5. A 50 µl volume was seeded for all experiments except for the thrashing and stress resistance assays, in which 100 µl was seeded.

For seeding NGM(-Glu), bacteria were no longer concentrated. Initially, a smaller volume was seeded; 75 µl of 168 MS, 150 µl of 168 Δ*atp* (corresponding to

Fig. 6. 6A and Fig. 6. 7B). Subsequently, throughout all experiments the volume was increased by 2.7 x (200 μl of 168 MS, 400 μl of 168 Δ*atp*). 200 μl of 168 Δ*atp* were seeded, left to dry, and the plate was reseeded with the same volume.

#### 6.3.6.2 Seeding NGM with *E. coli* OP50-1

Where *E. coli* was included as a control diet during an experiment, 100 µl were seeded from a 4 °C stock culture onto NGM(-Glu) and left to grow for two days at RT. NGM(+Glu) plates were seeded with a 1/10 dilution of *E. coli* stock kept at 4 °C.

### 6.3.7 Imaging of *B. subtilis* spores

Spores were imaged as described in Chapter 4.2.7.

### 6.3.8 Nematode experimentation

Nematodes were synchronised and introduced as L1s onto plates seeded with the relevant diet as described in section 2. 2, Chapter 2. Nematodes in all experiments with *B. s.* 168 MS or 168 Δ*atp* developed in the absence of glucose until the L4 stage. At this point, they either continued to be maintained on NGM(-Glu) or were transferred onto NGM(+Glu) for the remainder of the experiment, on the same diet (as previously).

Nematodes were maintained at a density of 15-18 individuals per assay plate throughout all experiments. Animals were transferred onto freshly seeded NGM plates every other day.

#### 6.3.8.1 Lifespan assays

Lifespan assays were conducted as previously described in section 3.3, Chapter 3.

#### 6.3.8.2 Pumping rate measurements

The rate of pharynx pumping ($min^{-1}$) was calculated as described in section 4. 3, Chapter 4.

#### 6.3.8.3 Body size measurements

Nematodes were synchronised and introduced onto each diet as described. Images were taken at the indicated day of adulthood. Before imaging, 14-17 adults were picked onto an empty NGM plate, immobilised with 0.5 M sodium azide, and aligned with a platinum wire pick. The body area was measured using ImageJ (ver. 2.0.0), using the wand tool. Two independent trials were performed.

#### 6.3.8.4 Oxidative stress resistance assay

Nematodes were synchronised and introduced onto each diet as described. At the indicated adult timepoints, 15 animals were transferred onto two 35 mm plates containing unseeded NGM(-Glu) (30 animals in total). Smaller plates were used to limit the volume of paraquat solution required. A 100 μl droplet of 500 mM paraquat solution (in $_{dd}H_20$) was pipetted onto the top of these nematodes to initiate the assay. Plates were checked every hour for seven hours, and the number of dead individuals was recorded. Animals were scored following gentle prodding of the nematode head with a platinum wire pick. The assay was repeated three times.

#### 6.3.8.5 Heat resistance assay

Synchronised *C. elegans* L4 larvae were transferred onto each of the four experimental diets. At the indicated timepoints, 30 individuals fed on *B. s.* 168 MS or 168 Δ*atp* in the presence or absence of glucose were moved into a 37 °C incubator with stable temperature control. The duration of heat shock was reduced for each subsequent timepoint, to ensure a survival differential (older animals were more susceptible to heat shock). Heat-shocked animals were left to recover for 16 hrs at 20°C, and survival was scored with a platinum wire pick. The assay was repeated four times independently.

#### 6.3.8.6 Thrashing assay

The thrashing assay was performed as described in section 3.3, Chapter 3.

#### 6.3.8.7 DAF-16::GFP localisation

*C. elegans* strain TJ356 were synchronised and introduced onto *B. s.* 168 MS, 168 Δ*atp* or *E. coli* OP50 as described. DAF-16::GFP localisation in day 5 adults was quantified. Animals were immobilised in 0.5 M sodium azide on an empty NGM plate. Control animals were exposed to 37 °C for 15 min. Nematodes were imaged at 63x magnification. 20-30 animals were imaged for each condition.

### 6.3.9 Microscopy

A Leica DMi8 inverted microscope was used to screen for spores following deletion of *spoIIE.* Lifespan and thrashing measurements were made with an Olympus SZ61 stereo microscope. Fluorescent reporter expression was observed, and images were taken, using a Carl Zeiss Axio Zoom.V16 Fluorescence zoom microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) with GFP filter enabled (excitation: 470/40nm; emission: 525/50nm). Images for lawn growth, nematode development and body size were taken with the DIC setting.

### 6.3.10 Statistical analysis

Lifespan data were analysed with the log rank test and Holm-Sidak multiple correction for *p*-values, as described in Chapter 3. The data for nematode survival following paraquat treatment were analysed by the same method.

Differences in body area, pharynx pumping, thrashing and heat shock survival were assessed using a One-way ANOVA followed by Tukey's HSD *post-hoc* test, or a Welch ANOVA followed by the Games-Howell *post-hoc* test if variances were unequal.

DAF-16::GFP localisation was compared using Fisher's Exact test with Monte Carlo simulation and false discovery rate (FDR) *p* value adjustment.

A summary of experimental conditions, hypotheses and conclusions is given in Table 6. 2.

**Table 6. 2 Summary of conditions for the experiments in Chapter 6.** All bacteria were unconcentrated. Shaded cells indicate experiments in which a larger quantity of food (2.7x) was seeded onto NGM(-Glu) plates (see above).

| Figure | Hypothesis / question | Bacterial strains | *C. elegans* culture conditions | | Growth* | Conclusion |
|---|---|---|---|---|---|---|
| | | | L1-L4 | Adulthood | | |
| 6.6A | Differences in spore formation underlie the effects of ATP-deficient *B. subtilis* on lifespan. | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Spore formation is not required for *B. s.* 168 Δ*atp* to increase lifespan. |
| 6.6B | Extension of lifespan by 168 Δ*atp*(-Glu) was a result of food shortage and DR. | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu | 24 hrs | DR is likely to explain the majority of longevity elicited by *B. s.* 168 Δ*atp*(-Glu). |
| 6.7B | Did diet affect the location of *C. elegans* on the NGM plates? | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Roaming of *C. elegans* across bare agar was higher in animals fed on *B. s.* Δ*atp* during early adulthood. |
| 6.7C | High roaming behaviour of *C. elegans* fed on *B. s.* Δ*atp*(-Glu) reflects shortage of food | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Roaming on lawns of *B. s.* 168 Δ*atp*(-Glu) was reduced when more food was supplied. |
| 6.8 | Is pharyngeal pumping rate affected by bacterial diet? | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Pumping was unaffected by the mutant diet +Glu and was increased -Glu during early adulthood. |

| Figure | Hypothesis / question | Bacterial strains | *C. elegans* culture conditions | | Growth* | Conclusion |
|---|---|---|---|---|---|---|
| | | | L1-L4 | Adulthood | | |
| 6.9 | Is body size reduced by *B. s.* 168 Δ*atp*? | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Body size was slightly reduced by the mutant diet in early adulthood. |
| 6.10 | Healthspan is extended by *B. s.* 168 Δ*atp* | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | *B. s.* 168 Δ*atp*(-Glu) enhanced motility particularly. |
| 6.11 | Stress resistance is enhanced in *C. elegans* fed on *B. s.* 168 Δ*atp* | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Resistance to oxidative stress was enhanced in animals fed on 168 Δ*atp* without glucose only. |
| 6.12 | | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Resistance to heat stress was slightly enhanced in animals fed on 168 Δ*atp*(-Glu). |
| 6.13 | *B. s.* 168 Δ*atp* stimulates DAF-16 nuclear localisation | *B. s.* 168 MS<br>*B. s.* 168 Δ*atp* | -Glu | -Glu<br>+Glu | 24 hrs | Localisation of DAF-16::GFP in the nucleus was slightly higher in animals fed on *B. s.* 168 Δ*atp.* |

*, the length of time in which bacteria were left to grow on NGM prior to the introduction of nematodes at 20 °C.

## 6.4 Results

### 6.4.1 Spore formation and ATP synthase were deleted in *B. subtilis* 168

A spore-less *spoIIE* mutant was generated in the standard tryptophan-deficient (*trpc2*) laboratory strain, *B. subtilis* 168, which is amenable to genetic manipulation. The sporulation protein SpoIIE is not required during vegetative growth but plays a key role in sporulation by activating the transcription factor $\sigma^F$, as well as generating the septum separating the pre-spore from the mother cell (Carniol *et al.*, 2005). As a result, *B. subtilis* with a deletion of SpoIIE are unable to form spores but are unaffected in their vegetative cycle. Absence of spore formation was confirmed by visual screening following growth of transformed 168 MS in Difco Sporulation Medium (DSM) (see Methods).

This spore-less strain is referred to herein as *B. subtilis* 168 master strain (168 MS). Subsequently, the ATP synthase operon was deleted in this master strain to generate strain 168 Δ*atp*. Deletion of ATP synthase in 168 MS was confirmed by PCR using primers 1 and 2 in Table 6. 1 (Fig. 6. 1).

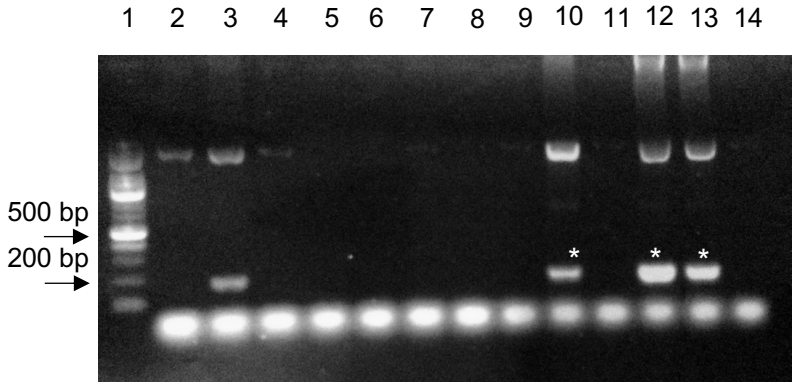



**Figure 6. 1 Confirmation of *atpA-I* deletion in *B. s.* 168 MS to generate 168 Δ*atp*.** 168 MS was transformed with DNA to delete *atpA-I*. Single colony PCR was conducted on nine positive transformants which grew on 5 μg/ml phleomycin plates following transformation (annealing temperature of 55 °C as determined by gradient PCR). Tradis-F and pRMC_ATPase_Rw primers (1 and 2, Table 6. 1) were used to confirm deletion of *atpA-I*. Lane 1, purple 100 bp DNA ladder (NEB); lane 2, 168 parental (spore-forming); lane 3, template DNA of the regions flanking *atpA-I* (positive control for successful deletion of *atpA-I*); lane 4, a variant of the control template in lane 3; lane 5, negative control lacking any template DNA; lanes 6-14, template DNA from nine positive colonies following transformation of 168 MS to delete *atpA-I*. The expected band size was 200 bp (asterisks).

The absence of endospores in 168 MS and 168 Δ*atp,* in comparison to spore-forming 168 parental, is shown in Fig. 6. 2.

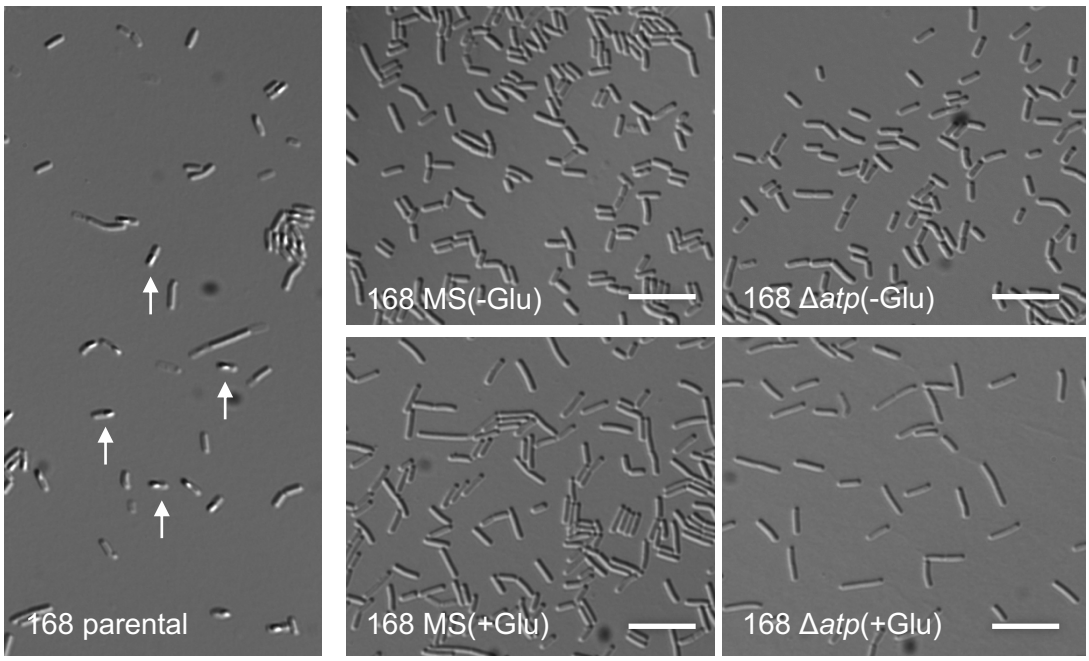



**Figure 6. 2 Endospore formation is absent in *B. subtilis* 168 lacking *spoIIE* (168 MS and 168 Δ*atp*).** Cells were removed from NGM plates after two day's growth at 20 °C. Endospores of the parental 168 strain (*spoIIE*+) were abundantly present (white arrows). Only vegetative growth was observed in 168 MS and 168 Δ*atp* (-/+ glucose). Scale bar = 10 μm.

### 6.4.2 Matrix formation was reduced in *B. subtilis* 168 MS and 168 Δ*atp*

As previously described, a higher proficiency for extra-cellular matrix formation is hypothesised to be important for the pro-longevity effects of *B. subtilis* lacking ATP synthase. A comparison of the growth of *B. s.* MS and Δ*atp* on NGM(+Glu) versus 168 MS and 168 Δ*atp* after 36 hrs at 20 °C is given in Fig. 6. 3A. Both 168 MS and 168 Δ*atp* developed into looser lawns that could be resuspended in liquid. Although with additional growth, a matrix formed over the surface of the central lawns of both strains, this was never as extensive as was observed for *B. s.* MS and Δ*atp.* As a result, the problem of matrix overgrowth was minimised in this strain.

Moreover, as shown in Fig. 6. 3B, larvae tended to reside within the central lawn of both 168 MS and 168 Δ*atp,* as opposed to on thinner patches of bacterial growth around the agar surface. This was true even on lawns of 168 Δ*atp*(+Glu), in contrast to what was observed in lawns of Δ*atp*(+Glu). There was therefore no

indication that *C. elegans* showed avoidance to 168 Δ*atp*(+Glu) lawns as larvae. It should be noted that, while the effect of glucose on development was investigated here, animals were consistently fed in the absence of glucose throughout larval development in subsequent experiments.

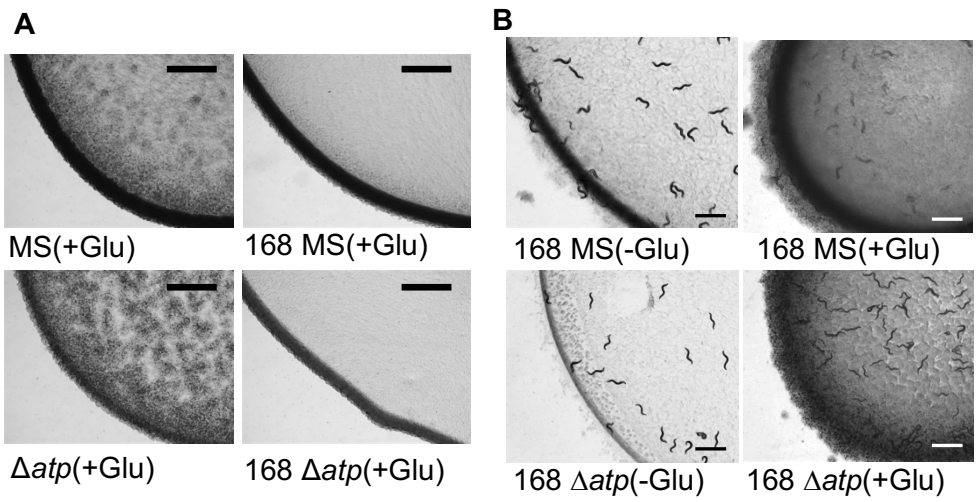



**Figure 6. 3 Lawn morphology was altered in *B. s.* 168 MS and Δ*atp* (A), and *C. elegans* larvae resided within the central lawn of each diet (B).** (A) Lawn growth and extracellular matrix formation in *B. s.* 168 MS(+Glu) and *B. s.* 168 Δ*atp*(+Glu) (right) was less extensive than in *B. s.* MS(+Glu) and Δ*atp*(+Glu). Images were taken after 36 hrs growth on NGM(+Glu) plates. Scale bar = 1 mm. (B) Location of L4 larvae on each bacterial lawn 48 hrs after introduction of L1s onto the plates. Animals were found within the central lawn on each of the diets, or in the border of the lawn. Scale bar = 1 mm.

Although matrix formation took a longer time to become apparent in *B. s.* 168 MS and Δ*atp* in comparison to the previous strains, by the point of nematode transfer away from the plates (72 hrs of lawn growth), a matrix had formed on both lawns in the presence of glucose. However, this matrix was confined to the border of the 168 MS(+Glu) lawn, whereas, as with spore-forming *B. s.* Δ*atp,* the entire surface of the mutant lawn gradually became encased (Fig. 6. 4).

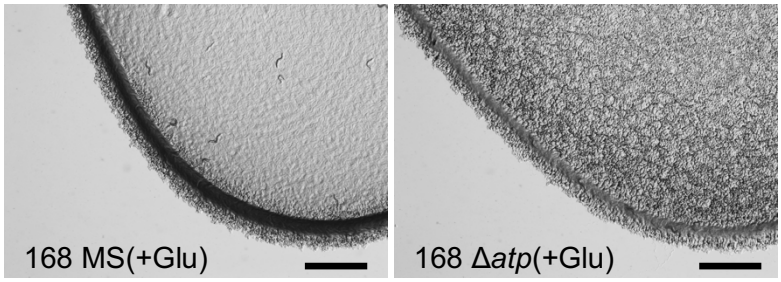



**Figure 6. 4 Growth of *B. subtilis* 168 MS and 168 Δ*atp* on NGM(+Glu) for 72 hours.** Bacteria were seeded and left to grow at 20 °C for 72 hrs. An extracellular matrix formed over the surface of 168 Δ*atp*(+Glu) lawns, but not 168 MS(+Glu). Scale bar = 1mm.

### 6.4.3 *B. s.* 168 Δ*atp* did not delay post-embryonic development

Previously, a diet of *B. s.* lacking ATP synthase elicited a developmental delay in *C. elegans. C. elegans* were synchronised and introduced onto lawns of the spore-less 168 MS or 168 Δ*atp* strains as L1 larvae. There was no apparent developmental delay when *C. elegans* were fed on 168 Δ*atp;* 48 hrs after the introduction of L1s onto each of the diets, the large majority of nematodes had entered into the mid/late L4 stage (Fig. 6. 5). This indicates that lack of ATP synthase is not in itself sufficient to delay development in *C. elegans.*

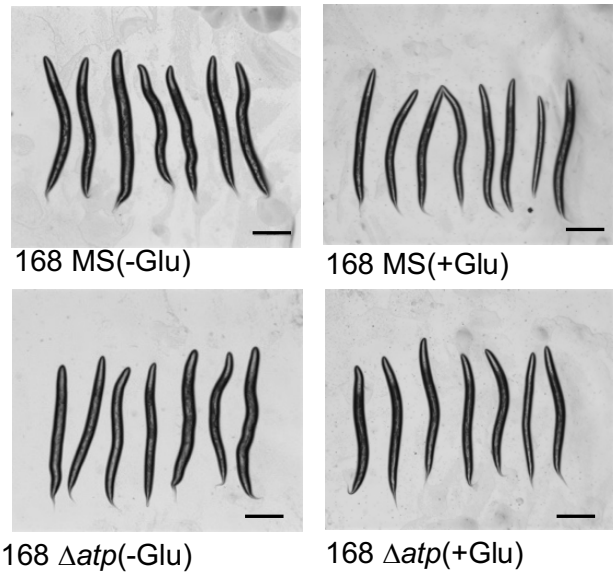



**Figure 6. 5 Development of *C. elegans* at 20 °C was similar on *B. s.* 168 MS and 168 Δ*atp*.** Representative images of *C. elegans* fed on each of the *B. subtilis* 168 diets in the absence (-Glu) or presence (+Glu) of glucose. Images were taken 48 hrs after introduction of L1s onto the indicated diet (50 x magnification). Development was not delayed by feeding on the ATP synthase mutant, with most animals having synchronously entered mid/late L4. Scale bar = 200 μM.

### 6.4.4 *C. elegans* lifespan was extended by *B. s.* 168 Δ*atp*(-Glu) and 168 Δ*atp* (+Glu)

In Chapter 3, a diet of *B. s.* lacking ATP synthase was shown to increase the lifespan of *C. elegans,* only when glucose was present (in the absence of FUdR). To investigate whether mutation of this gene in spore-less bacteria of the parental 168 genetic background was still able to exert this effect on *C. elegans,* the lifespan of N2 animals fed on 168 MS or Δ*atp* in the absence and presence of glucose was measured (Fig. 6. 6).

As described, concentrating the bacterial lawn induced UPR$^{mt}$ in *C. elegans,* as shown by upregulation of *Phsp-6::gfp* (Chapter 3). Therefore, the bacteria were not concentrated prior to seeding in the absence of glucose but were instead seeded directly onto NGM(-Glu).

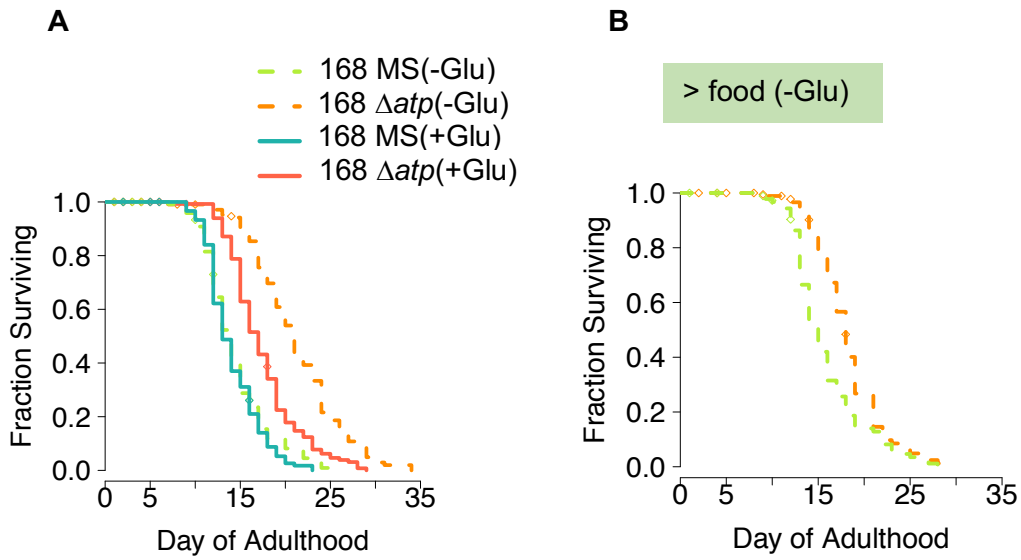



**Figure 6. 6 *B. subtilis* 168 Δ*atp* extended the lifespan of *C. elegans* with and without glucose (A) but longevity without glucose varied with food quantity (B).** (A) Lifespan of N2 individuals fed on *B. s.* 168 MS or 168 Δ*atp*(-Glu) or +Glu was monitored. Nematodes fed on *B. s.* 168 Δ*atp* lived longer than 168 MS-fed individuals both with and without glucose ($p < 0.0001$, log rank test). However, nematodes fed *B. s.* 168 Δ*atp*(-Glu) were the longest lived, showing an increase in mean lifespan of around 40% compared to those fed *B. s.* 168 MS(-Glu) ($p < 0.0001$, log rank test). The experiment was conducted over two independent replicates using a total of 220-270 individuals in each condition for both replicates. The graph is representative of both experiments. (B) Lifespan assay with the addition of 2.7x as much food in the absence of glucose (> food (-Glu)). Upon addition of a greater volume of *B. s.* 168 Δ*atp* to the NGM(-Glu) plates (400 μl, compared with 150 μl in A) the magnitude of lifespan extension was reduced to 16% (mean). The difference in the survival curves was, however, still significant ($p = 0.0003$, log rank test). Data were collected from a single replicate (N=94-100). All lifespan experiments were conducted at 20 °C. Diamond marks represent censoring. Log rank test with Holm-Sidak test for multiple comparisons on Kaplan Meier survival curves.

Glucose failed to shorten the lifespan of nematodes fed on *B. s.* 168 MS ($p = 0.31$, log rank test, N = 119-122) (Fig. 6. 6A). Consistent with previous results, 168 Δ*atp*(+Glu) extended lifespan compared to animals fed on 168 MS(+Glu) ($p < 0.0001$, log rank test, N = 121 and 122 respectively). The magnitude of the increase in mean lifespan is similar to that seen using the previous spore-forming strain (around 20%). Surprisingly, however, 168 Δ*atp*(-Glu) (N = 102) extended mean lifespan by an even greater amount (48%) in comparison to animals fed on 168 MS(-Glu) (N = 119) ($p < 0.0001$, log rank test) (Fig. 6. 6A). This experiment was repeated with the same results (Appendix Fig. D1, Table D1). 168 Δ*atp* therefore extended lifespan even without glucose.

### 6.4.5 Changing the quantity of 168 Δ*atp*(-Glu) altered nematode lifespan

Lawns of respiratory-deficient *B. s.* 168 Δ*atp* grew more poorly than 168 MS, particularly in the absence of glucose. Therefore, one trivial explanation for longevity of *C. elegans* fed on *B. s.* 168 Δ*atp*(-Glu) lawns is that food levels were insufficient, and nematodes underwent DR. Supporting this interpretation, a large proportion of nematodes fed on 168 Δ*atp*(-Glu) were censored (around 40% in two independent trials), primarily as a result of escape from the plate (Appendix Table D1). This perhaps gives an indication that food was limited in this group. Escape behaviour was also elevated in 168 Δ*atp*(+Glu)-fed individuals (Appendix Table D1).

Initially, a total of 150 µl of 168 Δ*atp*(-Glu) was seeded. If DR as a result of insufficient lawn growth was responsible for the longevity of nematodes fed on this diet, increasing the volume of bacteria seeded should alleviate this. Therefore, a lifespan experiment in which plates were seeded with a total of 400 µl (200 µl x 2) of 168 Δ*atp*(-Glu) (2.7x the previous quantity) was undertaken. The volume of 168 MS that was seeded was increased by the same factor, to 200 µl. whilst maintaining the same number of nematodes on each plate. The result of this experiment is shown in Fig. 6. 6B.

With a larger lawn of food, 168 Δ*atp*(-Glu) was still able to significantly extend the lifespan of *C. elegans* ($p$ = 0.0003, log rank test). However, the magnitude of extension was only 16%. The majority of this difference arose as a consequence of the mean lifespan of animals fed on *B. s.* ∆*atp*(-Glu) falling by 17 - 20%, simply by providing more food. Maximum lifespan in animals fed on larger lawns of 168 MS (24.44 days, N = 94) was very similar to that of animals fed on 168 ∆*atp.* (25.10 days, N = 100). In contrast, when the bacterial lawns were smaller (Fig. 6. 6A), the maximum lifespan of animals fed on 168 Δ*atp*(-Glu) was 28 days; 34% longer than individuals fed on 168 MS(-Glu). Therefore, providing more food completely prevented 168 Δ*atp*(-Glu) from increasing maximum lifespan.

Strikingly, no individuals escaped from the NGM(-Glu) plates when a greater quantity of food was provided (Appendix Table D1), and vulval explosion was consistently low under all conditions.

### 6.4.6 Supplying more food to *C. elegans* -Glu reduced roaming behaviour

Once again, the location of *C. elegans* in different 'zones' of agar plates seeded with *B. s.* 168 MS or 168 Δ*atp* was quantified. Data were collected on days 2 and 7 (and 11 as indicated) of adulthood, throughout the course of the lifespan experiments presented in Fig. 6. 7. See Appendix Table D2 and D3 for data and statistics.

The data depicted in Fig. 6. 7B shows the percentage of nematodes in the different zones when a smaller quantity of food was provided in the absence of glucose (corresponding to the lifespan experiment in Fig. 6. 6A). The location of 170-250 individuals fed on each of the four diets in two independent trials was recorded on days 2, 7 and 11 of adulthood. Under these conditions, nematodes fed on *B. s.* 168 Δ*atp*(-Glu) had considerable longevity. On day 2, 7 and 11 of adulthood, (Fig. 6. 7B), diet had a statistically significant effect on nematode location on the plate ($\chi 2$ = 547.607, 616.159, 172.764 respectively, $p$<0.0001). While only a small number of nematodes fed on *B. s.* 168 MS, with or without glucose, were found in zone 4 on the second day of adulthood (< 2%), 44% of 168 Δ*atp*(-Glu)-fed individuals and 27% of those fed 168 Δ*atp*(+Glu) were found roaming the plate outside of bacteria. Nematodes fed on 168 MS were distributed fairly evenly between different bacterial zones in the absence of glucose, whereas the vast majority were located in zone 3 when glucose was present. A similarly large proportion (67%) of nematodes fed on *B. s.* 168 Δ*atp*(+Glu) were located within zone 3 on day 2 of adulthood. On day 7, only 2% of individuals fed on 168 Δ*atp*(-Glu) were found outside of bacteria, whereas the proportion remained high for those fed on 168 Δ*atp*(+Glu) (17%). By day 11 of adulthood, the majority of nematodes were located within the central lawn (68 - 80%). A common theme across the days was the relatively high proportion of nematodes fed on *B. s.* 168 Δ*atp*(+Glu) that were located within zone 3. On days 7 and 11, this proportion was low (< 3%) for the other diets, but high (58% and 23%, respectively) in animals fed on 168 Δ*atp*(+Glu).

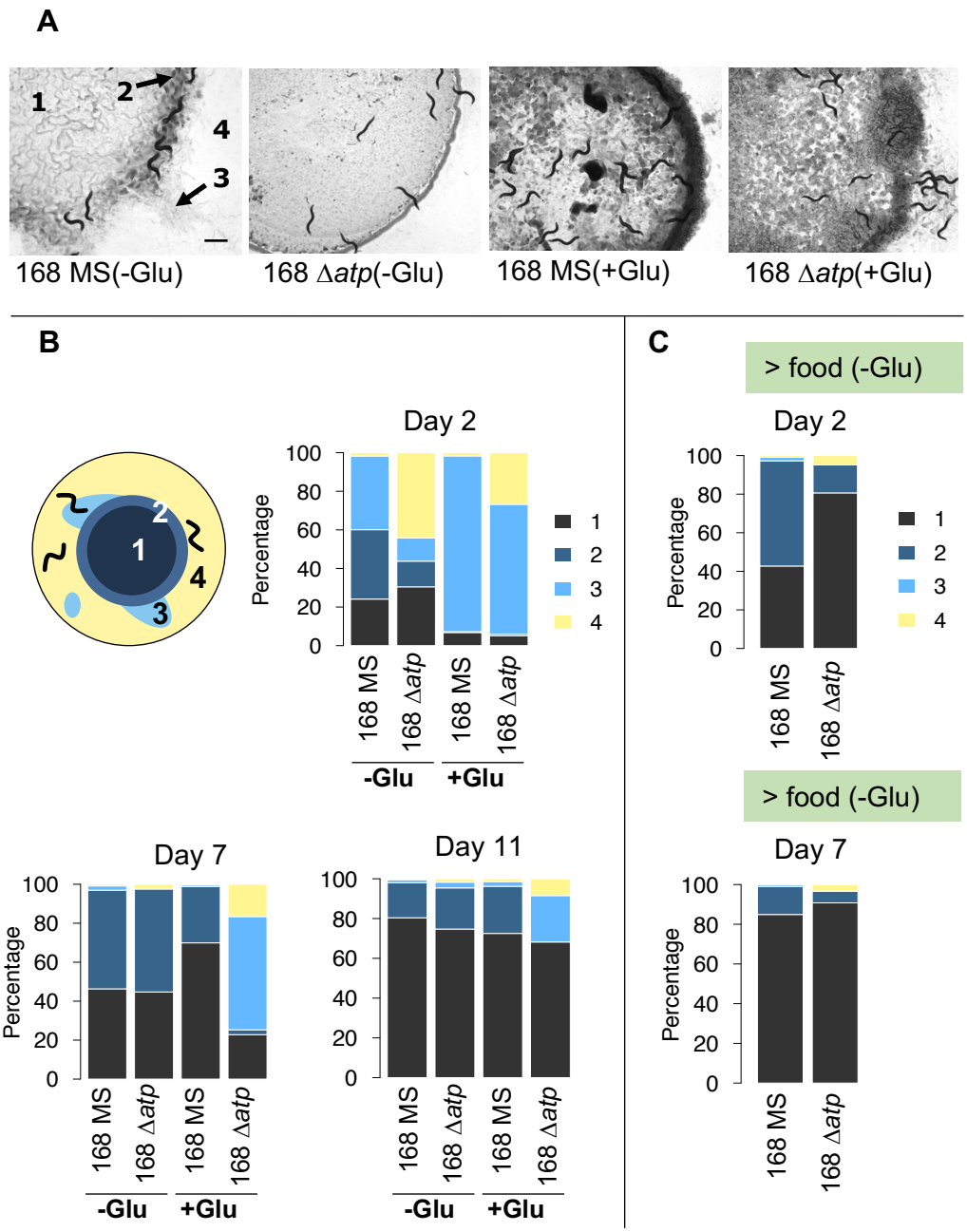



**Figure 6. 7 Analysis of nematode location on NGM seeded with *B. s.* 168 MS or 168 Δ*atp*.** (A) Four zones were defined based on the growth of the bacteria on the NGM (see main text). Day 5 adults are shown feeding on each of the diets. Scale bar = 1 mm. (B) Percentages of nematodes inside the different zones on days 2, 7 and 11 of adulthood. The number of individuals (N=170-250) in each condition found in each zone were averaged across two independent trials. Values are given as percentages of total individuals in each condition. (C) Percentages of nematodes in the different zones with more food (2.7 x the quantity) seeded onto NGM(-Glu) plates. Data from B and C were collected during the lifespan experiments shown in Fig. 6. 3A and 6. 3B, respectively. The location of 106-124 individuals fed on both diets was recorded at each timepoint. Location data on each day were compared using Pearsons' Chi-square test.

Larger lawns of bacteria were found to reduce the lifespan of nematodes fed on *B. s.* 168 Δ*atp*(-Glu) (Fig. 6. 6B). How this affected the location of nematodes (N=106-124) on the plate was investigated on day 2 and 7 of adulthood (Fig. 6. 7C). The relationship between diet and location was significant for day 2 adults ($\chi 2$ = 34.277, $p < 0.0001$), but not for day 7 adults ($\chi 2$ =1.305, $p = 0.253$). On day 2, this difference arose from there being a higher percentage of 168 MS(-Glu)-fed animals in zone 2 of the lawn (54.54%) compared with those fed on 168 Δ*atp*(-Glu)-fed (14.52%). However, on both diets, *C. elegans* was found predominantly within zones 1 and 2, and rarely outside of bacteria altogether (< 5%), on both day 2 and 7.

Increasing the quantity of food therefore had a striking effect on the location of nematodes on the plate. The proportion of individuals fed on 168 Δ*atp*(-Glu) inside the central bacterial lawn rose from 30% to 81% when more bacteria were seeded onto the plate (day 2). Moreover, on day 2 the percentage of 168 Δ*atp*(-Glu)-fed animals that were within some type of food (zone 1-3) increased from 56% to 95% following the addition of more food. The location data for nematodes fed with more food (Fig. 6. 7C) was directly compared to those in which less food was provided (Fig. 6. 7B), in the absence of glucose, with Pearson's Chi-square test (see Appendix Table D3). This confirmed that providing more food to *C. elegans* led to a significant reduction in the proportion of individuals outside of *B. s.* 168 Δ*atp* growth (i.e. in zone 4) on day 2 of adulthood ($\chi 2 = 98.6$, $p < 0.0001$). A larger quantity of food increased the proportion of nematodes within the central lawn of both *B. s.* 168 MS(-Glu) and 168 Δ*atp*(-Glu) on day 2 and day 7 ($p < 0.0001$).

Together with the observation that considerably fewer individuals fed on 168 Δ*atp*(-Glu) escaped when more bacteria were seeded, this is consistent with the hypothesis that food supply was previously insufficient. As a result, all subsequent experiments were carried out with this larger food quantity.

### 6.4.7 *B. s.* 168 Δ*atp*(-Glu) stimulated pharyngeal pumping

The rate of pharyngeal pumping can indicate the quantity of food intake, as well as the muscular health of *C. elegans* as it ages. The frequency of pharynx pumping in one minute was measured in 20-24 individuals fed on *B. s.* 168 MS or 168 Δ*atp* across two independent trials (Fig. 6. 8). Day 1, day 4 and day 15 adults were assessed. Outliers were excluded from statistical analysis, and data points

representing animals that were roaming across the bare agar (in which pumping rate fluctuated as a result of location on the plate) are encircled in Fig. 6. 8 (see Appendix Table D4 and D5).

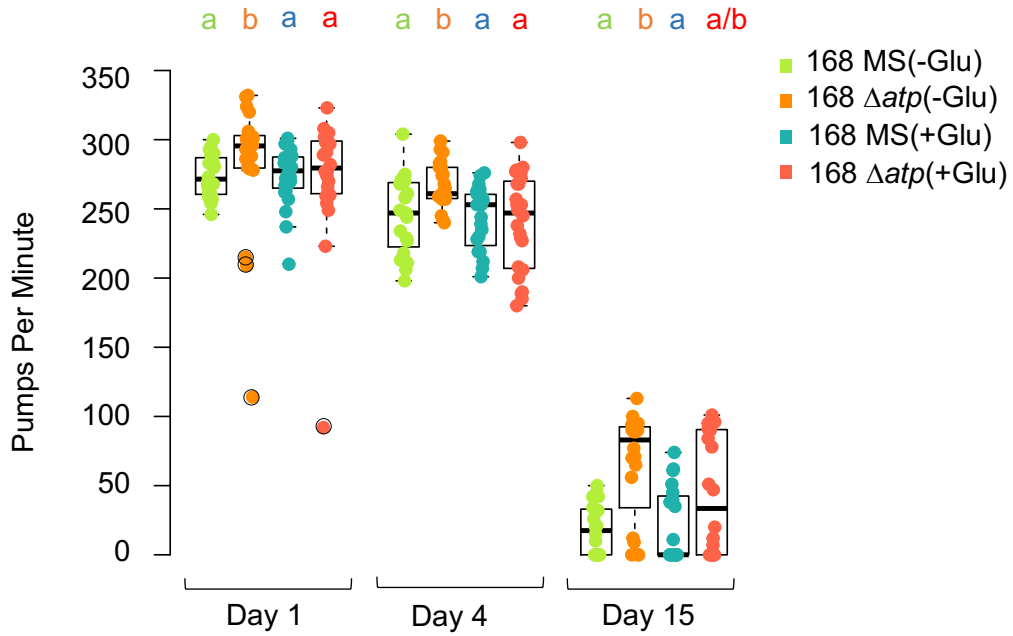



**Figure 6. 8 *B. subtilis* 168 Δ*atp* did not reduce the rate of pharyngeal pumping in *C. elegans*.** Pharyngeal pumping over a one-minute period was measured in individuals at day 1, day 4 and day 15 of adulthood, in two independent trials (20-24 animals in total). Different letters indicate significant differences between diets on the same day. On each day, pumping rate was unaffected by *B. s.* 168 Δ*atp*(+Glu) but was increased by this diet when glucose was absent. Data were analysed with a Kruskal-Wallis test with Dunn's FDR-corrected *post-hoc* test (day 15) or a one-way ANOVA, with Welch's correction for unequal variance for day 4 data, followed by Tukey's HSD *post-hoc* test (day 1) or the Games-Howell *post-hoc* test (day 4).

Significance was determined using a one-way ANOVA followed by Tukey's HSD *post-hoc* test (day 1), Welch ANOVA followed by the Games-Howell *post-hoc* test (day 4) or a Kruskal-Wallis test with Dunn's *post-hoc* test (day 15). There was a significant effect of diet on pharyngeal pumping rate on day 1 ($F(8,88) = 8.0012$, $p < 0.0001$), day 4 ($F(3,48.506) = 9.7565$, $p < 0.0001$) and day 15 ($\chi 2 = 17$, $p = 0.0007$) of adulthood.

Feeding on 168 Δ*atp* did not reduce the rate of pharyngeal pumping. The average pumping rate of animals fed on *B. s.* 168 Δ*atp*(+Glu) was not significantly different to that of 168 MS(+Glu)-fed individuals on day 1 ($p = 0.6416$), day 4 ($p = 0.9678$) or day 15 ($p = 0.1114$) of adulthood. Similarly, the presence of glucose had

no statistically significant effect on the rate of pharyngeal pumping in animals fed on 168 MS on day 1 ($p$ = 1), day 4 ($p$ = 0.9962) or day 15 ($p$ = 0.9056) of adulthood.

Interestingly, a diet of *B. s.* 168 Δ*atp*(-Glu) elicited a significant increase in the rate of pharyngeal pumping on each day. On day 1, the average rate of pumping in these animals (298.76 ± 16.71 $min^{-1}$) was 6% higher than that of individuals fed on 168 Δ*atp*(+Glu) (280.74 ± 23.25 $min^{-1}$) ($p$ = 0.0141), and 9% higher than in animals fed on 168 MS(-Glu). On day 4, the average pumping rate of *C. elegans* fed on *B. s.* 168 Δ*atp*(-Glu) (266.96 ± 15.89 $min^{-1}$) was again significantly higher than that of animals fed on *B. s.* Δ*atp*(+Glu) (239.92 ± 35.05 $min^{-1}$, 11%>) ($p$ = 0.0084) and animals fed on 168 MS(-Glu) (245.39 ± 27.49 $min^{-1}$, 9%>) ($p$ = 0.0122). Even at day 15, when a number of animals on each diet had stopped pumping, animals fed on 168 Δ*atp*(-Glu) had a higher mean pumping rate (65.30 ± 38.49 $min^{-1}$) than animals fed on *B. s.* 168 MS(-Glu) (18.45 ± 18.20 $min^{-1}$) ($p$ = 0.0018) or on *B. s.* MS(+Glu) (20.85 ± 26.52) ($p$ = 0.0018), though pumping in animals fed on 168 Δ*atp*(+Glu) (43.45 ± 42.93) was not significantly different ($p$ = 0.1181). This diet therefore stimulated greater activity of the pharyngeal muscles as well as delaying their age-related decline.

### 6.4.8 *B. s.* 168 Δ*atp* slightly reduced nematode body size in early adulthood

The effect of *B. s.* 168 MS and 168 Δ*atp* on body size was assessed (N=29-33) (Fig. 6. 9, Appendix Table D6).

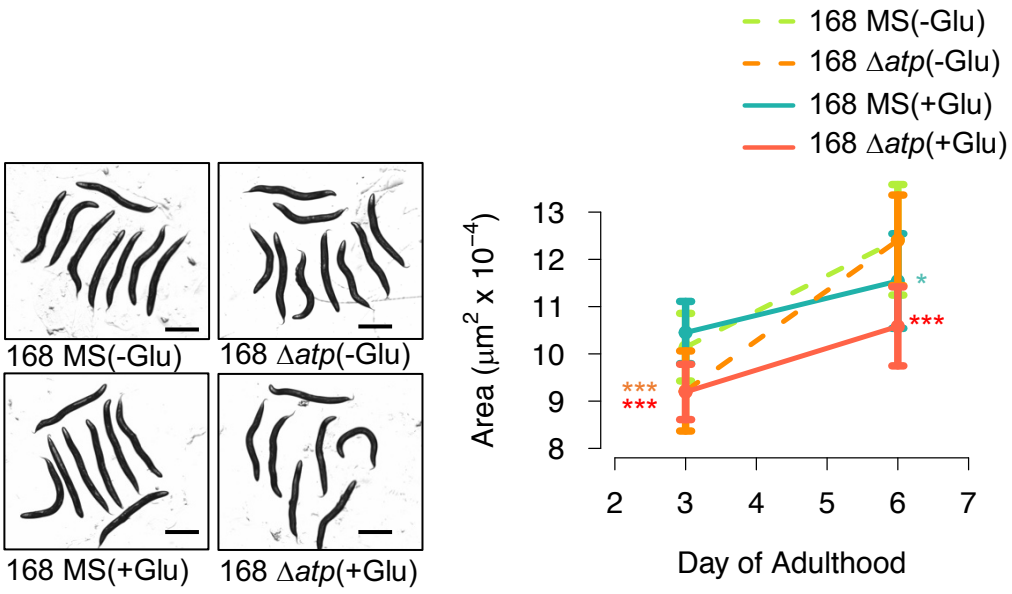



**Figure 6. 9 Body area of *C. elegans* was reduced by feeding on *B. subtilis* 168 Δ*atp*.** (A) Representative images of day 6 *C. elegans* adults fed on the indicated diets in the absence (-Glu) or the presence (+Glu) of glucose. Scale bar = 0.5 mm. (B) Quantification of *C. elegans* body area (μm$^2$) on days 3 and 6 of adulthood. Body area was lower in animals fed on 168 Δ*atp* on day 3, and in individuals fed on 168 Δ*atp*(+Glu) on day 6. Values represent the mean ± standard deviation of two independent replicates with 14-17 animals each. Asterisks indicate comparison to nematodes fed on *B. s.* 168 MS(-Glu). *, $p < 0.05$; ***, $p < 0.0001$, Tukey's HSD *post-hoc* test following one-way ANOVA.

There was a statistically significant difference between groups on day 3 as determined by one-way ANOVA ($F(3,124) = 26.661$, $p < 0.0001$). Tukey's *post hoc* test revealed that nematodes fed on *B. s.* 168 Δ*atp*(-Glu) (92,146 ± 8,471 μm²) were significantly smaller than those fed on 168 MS(-Glu) (101,415 ± 7,152 μm²), ($p < 0.0001$). Similarly, *B. s.* 168 Δ*atp*(+Glu)-fed individuals (91,946 ± 5,854 μm$^2$) were smaller than those fed on 168 MS(+Glu) (104,517 ± 6,580 μm$^2$) ($p < 0.0001$). The presence or absence of glucose had no significant effect on the body size of 168 MS-fed or 168 Δ*atp*-fed individuals ($p = 0.31$, $p = 1.0$, respectively).

On day 6, one-way ANOVA again revealed a significant difference between groups ($F(3,114) = 22.5709$, $p < 0.0001$). Nematodes fed on *B. s.* 168 Δ*atp*(-Glu)

were no longer smaller than those fed on *B. s.* 168 MS(-Glu) ($p$ = 1, Tukey's *post-hoc* test). However, 168 Δ*atp*(+Glu)-fed individuals were still significantly smaller than those fed 168 MS(+Glu) ($p$ = 0.0017, Tukey's *post-hoc* test) (reflecting an 8% reduction in mean body area). When nematodes were fed on 168 MS, body size was increased by 7% in the absence of glucose (124,136 ± 11,694 $\mu m^2$) compared to in its presence (115,416 ± 10,010 $\mu m^2$), ($p$ = 0.0058, Tukey's *post-hoc* test). Similarly, nematodes fed on 168 Δ*atp*(-Glu) were 15% larger (124,027 ± 9,581 $\mu m^2$) than those fed on 168 Δ*atp*(+Glu) (105,817 ± 8,354 $\mu m^2$), ($p$ < 0.0001, Tukey's *post-hoc* test). Therefore, by this timepoint, the presence of glucose reduced body size on both diets. While reduced, however, body size was not as severely affected by *B. s.* 168 Δ*atp*(+Glu) as by spore-forming *B. s.* Δ*atp*(+Glu) (Chapter 4).

### 6.4.9 Motility was increased by *B. s.* 168 Δ*atp* at a range of adult timepoints

In order to understand the effect of the spore-less *B. s.* 168 diets on health in *C. elegans,* motility in liquid medium was measured periodically through early-mid adulthood (N=40). Data were collected by a different researcher (Said, 2018). Diet significantly altered the rate of thrashing in liquid media on days 2 and 8 as determined by Welch ANOVA ($F$(3,85) = 53.6686, $p$ < 0.0001; $F$(3,86) = 57.8421, $p$ < 0.0001, respectively). On day 5 there was once again a statistically significant effect of diet on thrashing rate ($F$(3,156) = 71, $p$ < 0.0001, one-way ANOVA). The Games-Howell (day 2 and 8) or Tukey's (day 5) *post hoc* test was used for pairwise comparisons (see Appendix Table D7).

A diet lacking ATP synthase again increased the rate of thrashing (Fig. 6. 10). Thrashing was increased on this diet compared to *B. s.* 168 MS-fed individuals both with and without glucose. In comparison to nematodes fed 168 MS(-Glu), the mean thrashing rate of 168 Δ*atp*(-Glu)-fed animals on days 2, 5 and 8 was increased by 49%, 50% and 52% ($p$ < 0.0001). *B. s.* 168 Δ*atp*(+Glu) also increased thrashing compared to 168 MS(+Glu)-fed animals by 18%, 43% and 19% on days 2, 5 and 8 of adulthood, respectively ($p$ < 0.0001). When *C. elegans* was fed on 168 MS, the rate of thrashing was unaffected by the presence of glucose on days 2, 5 and 8 ($p$ = 0.98, $p$ = 0.55, $p$ = 0.91, respectively) of adulthood. However, nematodes fed on *B. s.* 168 Δ*atp*(-Glu) had a higher level of thrashing compared to those fed on 168 Δ*atp*(+Glu) (day 5, $p$ = 0.005, days 2 and 8, $p$ < 0.0001).

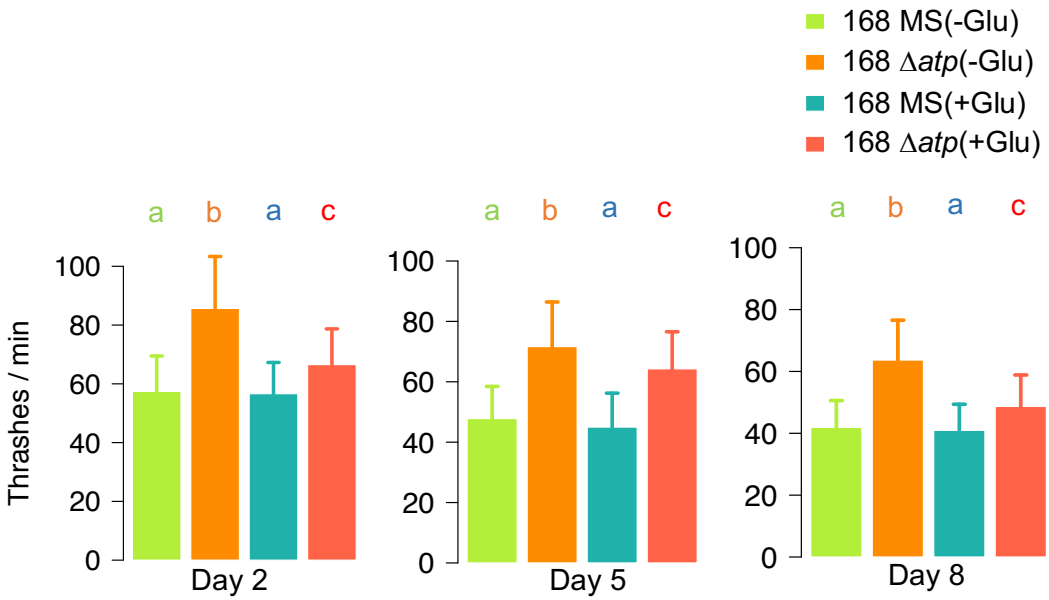



**Figure 6. 10 The thrashing rate in liquid of *C. elegans* fed on *B. s.* 168 Δ*atp* was increased throughout early-mid adulthood.** The thrashing rate (body bends/min$^{-1}$) of *B. s.* 168 Δ*atp* -fed nematodes was increased relative to those fed on 168 MS at each time point ($p < 0.0001$). When nematodes were fed on *B. s.* 168 Δ*atp*(-Glu), thrashing was increased relative to individuals fed on *B. s.* 168 Δ*atp*(+Glu) on day 2, 5 and 8 of adulthood ($p < 0.0001$, $p = 0.005$, $p < 0.0001$ respectively). The thrashing rate of *B. s.* 168 MS-fed individuals was unaffected by the presence of glucose on each day ($p > 0.05$). Values are the mean ± standard deviation of four replicates where 10 individuals were analysed per replicate. Different letters indicate significant differences in thrashing within each day. Welch ANOVA with Games-Howell *post-hoc* test (day 2 and 8); one-way ANOVA with Tukey's *post-hoc* test (day 5). Data were collected by Said (2018).

### 6.4.10 168 Δ*atp*(-Glu) enhanced resistance to abiotic stress

#### 6.4.10.1 Oxidative stress resistance was enhanced by *B. s.* 168 Δ*atp*

Following stresses including exposure to oxidative agents or temperature extremes, conserved cellular responses are activated to prevent and limit damage. Oxidative stress resistance varies depending on an organism's ability to detoxify reactive oxygen species (ROS) and repair damage to lipids, DNA and proteins (Senchuk *et al.*, 2017). Health and stress resistance are often positively associated. To test the hypothesis that stress resistance was enhanced in *C. elegans* fed on ATP synthase-deficient *B. subtilis,* resistance to paraquat-induced oxidative stress was measured in *C. elegans* fed on *B. s.* 168 MS or 168 Δ*atp* in the absence or the presence of glucose, at three adult timepoints (day 2, day 5 and day 8) (Fig. 6. 11). Stress resistance data (oxidative and heat stress) were collected by a different

investigator (Said, 2018). Data were analysed with a log-rank test on Kaplan-Meier survival curves (see Appendix Table D8 for descriptive statistics).

Mortality was reduced to a significant degree in nematodes fed on *B. s.* 168 Δ*atp*(+Glu) compared to those fed on 168 MS(+Glu) ($p$ <0.0001, log rank test) on days 2, 5 and 8. However, in the absence of glucose, *B. s.* 168 Δ*atp* induced a far more striking defence against oxidative stress on each day of adulthood ($p$ < 0.0001, log rank test). On day 2, 5 and 8, only 38%, 41% and 66% of 168 Δ*atp*(-Glu)-fed nematodes had died, respectively, by the end of the 7-hr observation period (N = 90). In comparison, 88% – 100% of animals fed on the other diets had died within 7 hrs on each day (N = 90). The presence of glucose only affected the survival of *C. elegans* fed on *B. s.* 168 MS on day 8, when individuals fed on 168 MS(+Glu) had reduced resistance to paraquat compared to those fed on 168 MS(-Glu) ($p$ = 0.0031, log rank test).

A diet of *B. s.* 168 Δ*atp* therefore enhanced resistance to oxidative stress induced by paraquat throughout early-mid adulthood, and this was particularly pronounced in the absence of glucose.

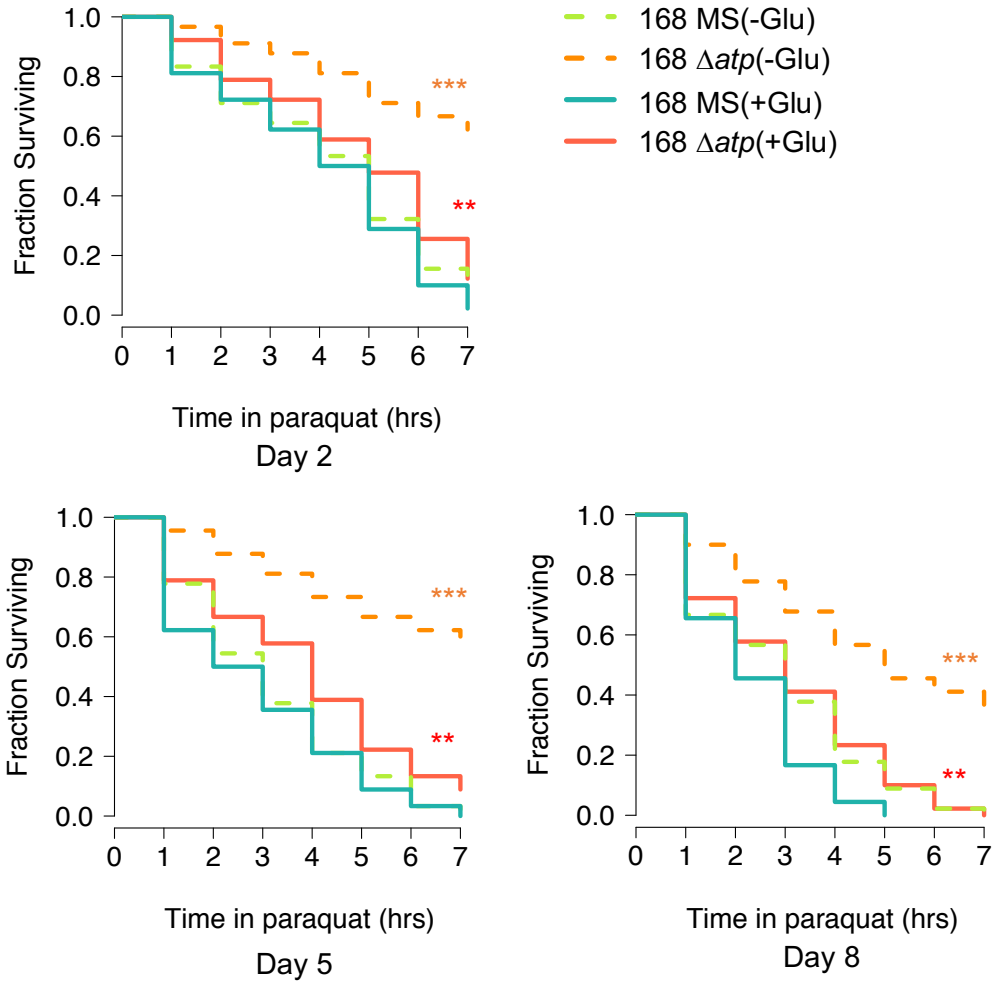



**Figure 6. 11 Survival of *C. elegans* following exposure to 500 mM paraquat over a 7-hour period.** *C. elegans* were exposed to 500 mM paraquat, and survival was monitored every hour. Individuals at day 2, day 5 and day 8 of adulthood were assessed. Feeding on *B. s.* 168 Δ*atp* enhanced resistance to oxidative stress on each day. This was more pronounced in those individuals fed on *B. s.* 168 Δ*atp*(-Glu). Asterisks next to 168 Δ*atp*(-Glu) (orange) and 168 Δ*atp*(+Glu) (red) survival curves indicate comparison to nematodes fed on 168 MS(-Glu) or 168 MS(+Glu), respectively. Curves represent the survival of 30 individuals pooled across three independent replicates (N = 90 per condition). Data were analysed with a log rank test on Kaplan-Meier survival curves, with Holm *p* value adjustment. **, $p < 0.001$; ***, $p < 0.0001$. Data collected by Said (2018).

#### 6.4.10.2 168 Δ*atp*(-Glu) enhanced resistance to heat during early adulthood

Upon the detection of increased temperature, the heat shock response (HSR) is activated by the conserved transcription factor HSF-1. HSF-1 induces the expression of heat shock proteins (HSPs) and molecular chaperones to refold and the prevent the accumulation of misfolded proteins (Kapahi *et al.*, 2017). Here,

resistance to heat shock at 37 °C was measured in *C. elegans* fed on each of the *B. s.* 168 diets (Fig. 6. 12, Appendix Table D9 and D10).

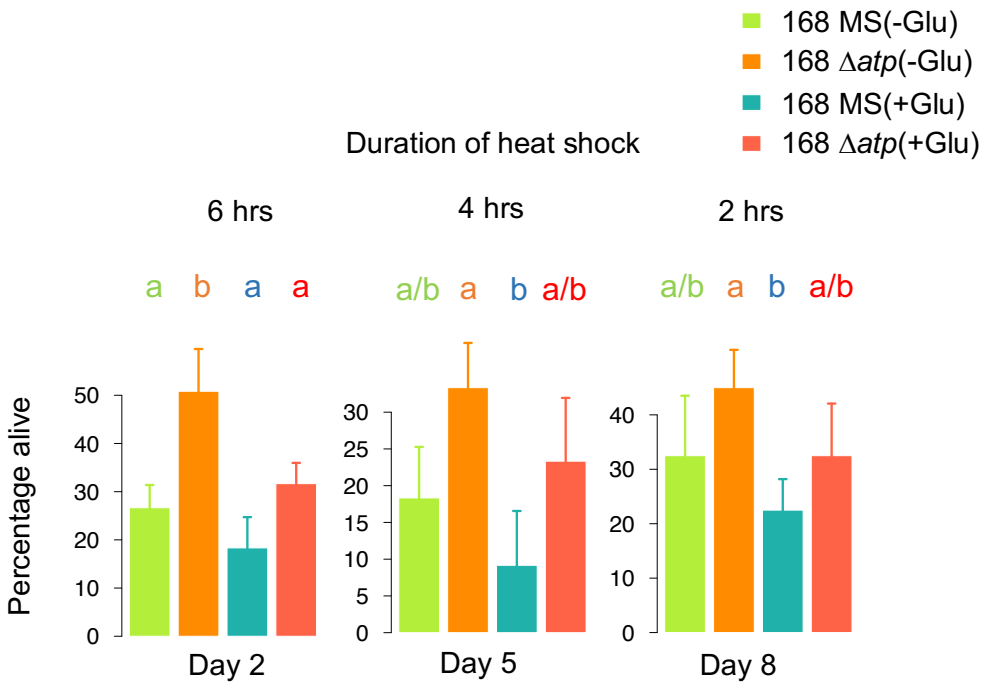



**Figure 6. 12 Survival of *C. elegans* following different durations of heat shock at 37 °C when fed on *B. s.* 168 MS or 168 Δ*atp* with or without glucose.** Day 2, day 5 and day 8 adults were subjected to 6 hrs, 4 hrs or 2 hrs of heat shock, respectively. Survival was enhanced in *C. elegans* fed on *B. s.* 168 Δ*atp* on day 2 of adulthood. Data represent the mean ± standard deviation of four biological replicates with 30 individuals per replicate. Different letters indicate significant differences in survival within each day (Welch ANOVA followed by the Games-Howell *post-hoc* test).

A Welch ANOVA revealed a significant effect of diet on heat stress survival on day 2 ($F(3, 6.5) = 10.619$, $p = 0.0066$), day 5 ($F(3, 6.6) = 7.529$, $p = 0.0152$) and day 8 ($F(3, 6.5) = 6.964$, $p = 0.0191$) of adulthood. The Games-Howell *post-hoc* test was used to determine pairwise significance on each day.

On day 2, animals fed on *B. s.* 168 Δ*atp*(-Glu) were more resistant to heat stress in comparison to those fed on 168 MS(-Glu) ($p = 0.0206$), 168 MS(+Glu) ($p = 0.0052$) and 168 Δ*atp*(+Glu) ($p = 0.0491$). Average survival was increased by 24%, 33% and 19%, respectively. There was no difference in survival between individuals fed on 168 MS(-Glu) compared to 168 MS(+Glu) ($p = 0.0588$). On days 5 and 8,

there was a significant difference in survival between animals fed on *B. s.* 168 MS(+Glu) and 168 Δ*atp*(-Glu) ($p$ = 0.01). However, the difference in survival between *C. elegans* fed on *B. s.* 168 MS(-Glu) and 168 Δ*atp*(-Glu) was not statistically significant on day 5 ($p$ =0.065) or day 8 ($p$ = 0.326). Therefore, although a trend of higher survival in *C. elegans* fed on *B. s.* 168 Δ*atp*(-Glu) is apparent from Fig. 6. 12, this only reached statistical significance on day 2.

### 6.4.11 *B. s.* 168 Δ*atp* induced nuclear localisation of DAF-16::GFP

Insulin signalling is a broadly conserved ageing-associated pathway in *C. elegans.* The transcription factor DAF-16 mediates downstream effects of this pathway by activating genes involved in stress resistance, immunity and longevity. Mutation of the insulin-like receptor, DAF-2, can increase nematode lifespan considerably by de-repressing DAF-16, which then translocates into the nucleus to modulate gene transcription. The influence of *B. s.* 168 MS and 168 Δ*atp* on the activation and localisation of DAF-16 (isoforms A/B) in *C. elegans* was therefore investigated. A transgenic *C. elegans* strain expressing a DAF-16::GFP fusion protein from the *daf-16* promoter was fed on each of the diets, and the number of individuals displaying cytoplasmic, intermediate or nuclear localisation of the reporter on day 5 of adulthood was quantified (Fig. 6. 13). See Appendix Table D11 for statistical analysis.

Positive control individuals which were fed on *E. coli* OP50-1(-Glu) and subjected to heat shock for 15 minutes at 37 °C displayed full DAF-16::GFP nuclear localisation. Pairwise Fisher's Exact Test were conducted with FDR $p$-value adjustment to determine significance for animals fed on *B. subtilis.* Nematodes fed on *B. s.* 168 MS only had cytoplasmic reporter localisation. In contrast, 22% of individuals fed on *B. s.* 168 Δ*atp*(-Glu) showed evidence of nuclear reporter localisation (nuclear and intermediate categories). This level of nuclear/intermediate localisation was significantly higher than what was observed in nematodes fed on 168 MS(-Glu) ($p$ = 0.0031) and 168 MS(+Glu) ($p$ = 0.0244). The pattern of reporter localisation in 168 Δ*atp*-fed individuals did not change significantly when glucose was present ($p$ = 0.2870). Despite this, nuclear/intermediate reporter localisation in individuals fed on 168 Δ*atp*(+Glu) was not significantly higher than that of animals fed on 168 MS(-Glu) ($p$ = 0.2426) or 168 MS(+Glu) ($p$ = 0.4422).

These results indicate that nuclear localisation of DAF-16 is induced by a diet of 168 Δ*atp*, exclusively or predominantly when glucose is absent. Although the effect was relatively mild, this implicates IIS signalling in the response of *C. elegans* to the mutant diet.

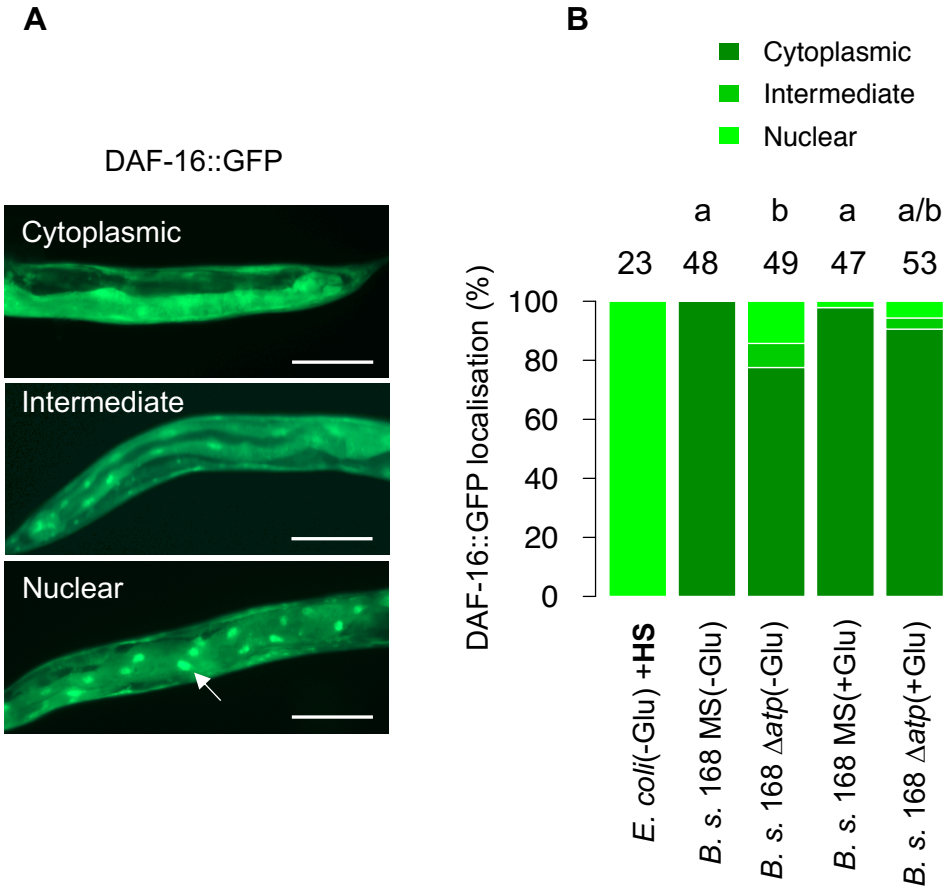



**Figure 6. 13 Localisation of DAF-16::GFP to intestinal nuclei was induced by a diet of *B. s.* 168 Δ*atp*(-Glu).** (A) Representative images of cytoplasmic, intermediate or nuclear DAF-16::GFP localisation in day 5 individuals. The arrow indicates nuclear localisation of DAF-16::GFP in an intestinal cell nucleus. Scale bar = 50 μm. (B) Feeding on *B. s.* 168 Δ*atp*(-Glu) induced nuclear localisation of DAF-16::GFP in comparison to all conditions except the heat shock control ($p < 0.01$). The increased frequency of DAF-16::GFP nuclear localisation in nematodes fed on *B. s.* 168 Δ*atp*(+Glu) was not statistically significant ($p < 0.05$). The same letter above the bars indicates no significant difference in reporter localisation. Numbers above the bars represent the total number of individuals screened across two independent replicates. Conditions were compared using Fisher's Exact Test (FDR *p* value adjustment).

## 6.5 Discussion

Here, a diet of spore-less *B. subtilis* lacking ATP synthase was shown to act as a probiotic when fed to *C. elegans.* The data collected in Chapter 3 began to indicate that, although it did not affect lifespan, a diet of *B. s.* Δ*atp* had beneficial effects on the health of *C. elegans.* The data presented here corroborate this.

### 6.5.1 *B. s.* 168 Δ*atp*(-Glu) enhanced the stress resistance and health of *C. elegans*

Similar to what was observed in Chapter 3 using the spore-less *B. subtilis* strains, motility was improved by the ATP synthase mutant. Clearly, therefore, sporulation is not a requirement for the ATP synthase-deficient diet to improve the health of *C. elegans.* The stress resistance of *C. elegans* fed on *B. s.* MS and Δ*atp* has not yet been measured. However, data is presented in this chapter which show that oxidative stress resistance was enhanced in *C. elegans* fed on the 168 ATP synthase mutant diet. Though the benefits of this diet were apparent whether glucose was absent or present, survival in the absence of glucose was particularly striking. There are many approaches to studying sensitivity to oxidative stress in *C. elegans* including exposure to paraquat (used here), arsenite, hydrogen peroxide, juglone and t-BOOH. Depending on the type of oxidative stressor, different subcellular compartments are affected and *C. elegans* will be exposed to different types of ROS (e.g. hydrogen peroxide, superoxide, hydroxyl radicals) (Senchuk *et al.*, 2017). Moreover, depending on the type of assay employed the exposure may be acute or chronic. As a result, the sensitivity of *C. elegans* to oxidative stress is likely to vary depending on the assay (Senchuk *et al.*, 2017). In future it will be informative to employ a wider range of approaches to better characterise the nature of oxidative stress resistance conferred by the mutant diet.

Resistance to heat stress was also enhanced by a diet of *B. s.* 168 Δ*atp*(-Glu). Improved survival elicited by this diet only reached statistical significance on day 2 of adulthood. However, the trend across the days indicates that a real biological effect might have been present at the later timepoints as well. A more sensitive assay might reveal this to be the case. For example, a longitudinal study in which survival is measured on the same individuals at periodic intervals (such as in the oxidative stress assay) would be informative. Interestingly, enhanced resistance to oxidative stress, imposed by the quinone juglone, as well as resistance to heat

stress was similarly observed in *C. elegans* fed on respiration-deficient *E. coli* lacking coQ (Gomez *et al.*, 2012).

Low levels of mitochondrial stress through electron transport chain inhibition have been shown to increase resistance to heat (Labbadia *et al.*, 2017) and oxidative stress (Dues *et al.*, 2017) with age. This is associated with maintained function of HSF-1, or elevated ROS and activation of *sod-3,* $UPR^{mt}$ (*Phsp-6::gfp*) and SKN-1 (*Pgst-4::gfp*)-mediated stress responses, respectively. Similarly, members of the natural *C. elegans* microbiome enhanced stress resistance and upregulated the $UPR^{mt}$ (Dirksen *et al.*, 2016). Enhanced stress resistance often appears to derive, therefore, from priming of stress responses. As described, however, none of these antioxidant and stress response genes were upregulated in animals fed on *B. s.* Δ*atp* in the absence of external stress (Chapter 3), although this was a different strain that formed spores and delayed nematode development, so any comparison should be drawn with caution. It would be interesting to determine whether such genes are similarly inactive in animals fed on *B. s.* 168 Δ*atp*(-Glu), to understand whether hormetic priming of stress responses contributes to stress resistance.

As discussed, despite its negative effects on lifespan, glucose released from glycogen stores in *C. elegans* enhances resistance to oxidants including paraquat and diamide (Gusarov *et al.*, 2017). Revealingly, diets of *B. s.* 168 MS(+Glu) did not enhance resistance of *C. elegans* to paraquat in longitudinal survival assays, compared to animals fed on 168 MS(-Glu) (Fig. 6. 11). In fact, by day 8 of adulthood the presence of glucose had become detrimental to nematode survival in 168 MS-fed populations, whereas 168 Δ*atp* only enhanced paraquat resistance when glucose was absent. This lends further support to the notion that *C. elegans* fed on *B. subtilis* were either not taking in glucose or were ingesting relatively low quantities – either passively as a consequence of removal from the agar, or actively by some protective component of *B. subtilis* metabolism.

These experiments demonstrate that ATP synthase-deficient *B. subtilis* is able to enhance health and stress resistance in *C. elegans.* Preliminary data are currently extending this research. DIC imaging of ageing nematodes indicates a striking delay in morphological decline as judged by reduced degeneration of tissue integrity throughout the body, and a reduction in the accumulation of fluid in the

pseudocoelom of *C. elegans* fed on *B. s.* 168 ∆*atp.* One currently unexplored question is whether *B. s.* ∆*atp* exerts an antimicrobial effect, a desirable feature of probiotic bacteria. Probiotics including *L. acidophila* and *P. mendocine* activate PMK-1/p38-dependent immune responses in *C. elegans* (Kim and Mylonakis, 2012; Montalvo-Katz *et al.*, 2013). As mentioned, *B. subtilis* GS67 protects *C. elegans* from *B. thurigiensis* through the production of fengycin (Iatsenko *et al.*, 2014). The resistance of *C. elegans* fed on the ATP synthase mutant against this pathogen, as well as the nematophagous fungal pathogens *Dipodascus* Myf82 (Dirksen *et al.*, 2016) and *Drechmeria coniospora* (Jansson, 1994), should be determined using longitudinal killing assays. Since brood size was found to be reduced by spore-forming *B. s.* ∆*atp*(-Glu) (Chapter 4), it will be important to measure reproductive health in *C. elegans* fed on *B. s.* 168 ∆*atp.*

### 6.5.2 The role of DR in the physiology of *C. elegans* fed on *B. s.* 168 Δ*atp*(-Glu)

Given the finding that some level of DR was induced in *C. elegans* fed on *B. s.* ∆*atp*(-Glu), it is particularly important to clarify the role of DR pathways in the benefits elicited by 168 Δ*atp*(-Glu). In all experiments with *B. s.* 168 MS and 168 Δ*atp* in this chapter, unconcentrated bacterial lawns were provided. Initially, 150 μl of the ATP synthase mutant were seeded onto NGM(-Glu), the same volume that was used with the previous strain (Chapters 3 and 4) and which did not increase lifespan. However, the mean lifespan of *C. elegans* fed on the same quantity of spore-less *B. s.* 168 Δ*atp* was 40-50% longer than that of animals fed on 168 MS(-Glu). As described, increasing the quantity of *B. s.* 168 Δ*atp* seeded onto NGM(-Glu) greatly reduced the magnitude of longevity induced by this diet. The simplest explanation for this phenomenon is that a greater quantity of food alleviated the effects of food shortage and DR, which accounted for most of the ~40% lifespan increase that was observed when less bacteria were seeded. Although nematodes did not deplete the food over the course of the experiment, it is possible that they experienced food shortages during the period of excessive progeny production in early adulthood when smaller lawns were provided. This conclusion is supported by the plate location data. Provision of more food substantially reduced roaming of *C. elegans* fed on *B. s.* 168 ∆*atp* across the unseeded agar, and lead to the vast majority of animals staying within zone 1 or 2 of the lawn without glucose. This is consistent with a dramatic reduction in the frequency of escape. This clearly indicates that the tendency of *C. elegans* to roam the agar was related to the quantity of bacteria on the plate.

In Chapter 4, *pha-4* was shown to be upregulated in *C. elegans* fed on spore-forming *B. s.* Δ*atp*(-Glu). The plates were seeded with the smaller volume (150 μl) of bacteria, and without concentrating – the same procedure that dramatically extended lifespan in the current chapter. It would seem likely that DR was being elicited through food shortage in this case. However, animals in which *pha-4* was upregulated were not long-lived. This can possibly be attributed to the use of concentrated bacterial lawns in many of these experiments (discussed in Chapter 4).

One obvious question that arises, if food shortage was indeed responsible for the longevity of animals fed on smaller lawns of 168 Δ*atp*(-Glu), is whether the addition of more bacteria alleviated this completely. When provided in greater quantity, 168 Δ*atp*(-Glu) was still able to extend lifespan, albeit to a reduced extent, and improved health and stress resistance in *C. elegans. C. elegans* undergoing solid DR or bacterial deprivation (BD) have enhanced resistance to paraquat (Greer *et al.*, 2007; Zhou *et al.*, 2011), although those subjected to liquid DR regimes do not. Similarly, growth in axenic culture or BD enhances nematode thermotolerance (Houthoofd *et al.*, 2002; Kaeberlein *et al.*, 2006). Pumping rate was actually higher in animals fed on 168 Δ*atp*(-Glu) than in all other conditions throughout early adulthood, and these animals had a clear tendency to remain within the central lawn instead of roaming the plate. Both of these behaviours were, however, true of animals fed on *B. s.* Δ*atp*(-Glu), in which *pha-4* was upregulated. Firstly, whether food intake is impaired should be investigated directly by using red fluorescent reporter strains of *B. s.* 168 MS and 168 Δ*atp,* which were also developed as part of this work. Preliminary data indicates that not only is food intake not reduced in animals fed on *B. s.* 168 Δ*atp*, it is actually slightly increased throughout early-mid adulthood. Similar to the approach in Chapter 4, determining the ability of *B. s.* 168 Δ*atp*(-Glu) to extend the lifespan of *eat-2* and *skn-1* mutants would be informative. Further approaches to investigating DR are detailed below.

### 6.5.3 Comparison of the effects of spore-forming and spore-less *B. subtilis* 168

The experimental conditions used in the current chapter compared to prior chapters were very similar. Bacteria were seeded onto NGM(-Glu) (often without concentrating in both cases) in similar volumes and the plates were left to grow for

the same period of time prior to introducing nematodes. NGM(+Glu) plates were prepared and seeded with the same protocol as previously. However, there were several differences in the growth and morphology of the bacteria in the parental 168 genetic background utilised here. Notably, deletion of *spoIIE* prevented bacterial sporulation in the lawns. Additionally, the onset of extracellular matrix formation in the bacterial lawns was delayed, seemingly as a consequence of a slightly reduced growth rate in comparison to the previous strain. Moreover, by several measures *C. elegans* responded in different ways to strains *B. s.* 168 MS and 168 Δ*atp,* in comparison to spore-forming strains *B. s.* MS and Δ*atp*. Notably, when *C. elegans* were fed on the spore-less 168 parental strain, the ATP synthase mutant no longer induced a developmental delay in the absence or presence of glucose. This shows that the delay that was previously seen using spore-forming ATP synthase mutant (Chapter 3) was not simply a response to respiration-deficient bacteria. Once again, animals fed on the ATP synthase mutant +Glu were smaller than those -Glu. However, the effect was considerably less severe in the case of the spore-less strains. At day 3, the mean body area of animals fed on *B. s.* 168 Δ*atp*(+Glu) was 9% less than those fed on 168 MS(-Glu); in contrast, *C. elegans* fed on the spore-forming ATP synthase mutant (Chapter 4) were 35% smaller (body volume) than those fed on *B. s.* MS(-Glu) at the same timepoint. It was previously postulated that the small body size of animals fed on *B. s.* Δ*atp*(+Glu) was a result of dietary restriction through feeding obstruction. Since the bacterial matrix was less extensive in lawns of the spore-less ATP synthase mutant, this hypothesis is consistent with a less dramatic reduction in body size in these animals as well as the absence of L1 arrest. Moreover, while day 3 adult *C. elegans* fed on the spore-forming ATP synthase mutant (Chapter 4) were the largest of any condition, animals of the same age that were fed on spore-less *B. s.* 168 Δ*atp*(-Glu) were amongst the smallest. Whether this reflects a state of DR is unclear in the absence of other physiological measurements such as fat content and fecundity.

It is possible that differences in the genetic background of the 168 strains was important. Spore-forming *B. subtilis* MS and Δ*atp* were derived from a strain, itself a 168 derivative, lacking a number of dispensable genomic regions, including the prophages SPβ and PBSX and the prophage-like element *skin* (Westers *et al.*, 2003). This amounts to loss of 231 kb of DNA relative to the parental *B. subtilis* 168 strain used in the current chapter. Deletion of ATP synthase in this background potentially gave rise to the observed phenotypic differences. Genetic differences

between strains might also change the micronutrient profiles of these diets, affecting nematode physiology, as in a recent report using *E. coli* (Han *et al.*, 2017). Precisely how these strain differences might have altered the physiology of *C. elegans* is currently unknown. However, the most notable difference between the strains is the sporulation capacity of the bacteria. It seems likely that the formation of spores by the ATP synthase mutant of this strain in earlier chapters contributed to delaying nematode development, as has been previously reported (Laaberki and Dworkin, 2008), and might also have contributed to some of the DR phenotypes of animals fed on *B. s.* Δ*atp*(+Glu), in combination with extensive matrix production. In future, direct comparison of the effects of spore-forming and spore-less diets in the same genetic background will be able to address this question. Deletion of ATP synthase in the spore-forming (*spoIIE*+) parental 168 strain and comparison to spore-less *B. s.* 168 Δ*atp*(+Glu) would be a facile approach.

### 6.5.4 *B. s.*168 Δ*atp* uncoupled lifespan from health and stress resistance

In spite of the differences in the results presented in this chapter compared to those using *B. s.* MS and Δ*atp,* there are some potentially revealing parallels. The lifespan of *C. elegans* was extended by *B. s.* 168 Δ*atp*(+Glu). The mean lifespan increase afforded by *B. s.* 168 Δ*atp* compared to the master strain was around 20%, very similar to that of animals fed on the previous *B. s.* Δ*atp* (although a much larger lifespan extension of 42% was recorded by a different investigator). The data presented in this chapter therefore show that the formation of spores is not a requirement for ATP deficient *B. subtilis* to increase lifespan. Similarly, glucose once again failed to reduce the lifespan of *C. elegans* fed on the master strain.

Moreover, it seems that beneficial effects of the respiration-deficient diet on health (and, in the case of strain 168, stress resistance), can once again be uncoupled from longevity. When a greater quantity of *B. s.* 168 Δ*atp*(-Glu) was provided to *C. elegans,* longevity in comparison to 168 MS(-Glu)-fed individuals was reduced dramatically, although not abolished completely. However, the same quantity of food was able to greatly improve motility and oxidative stress resistance with age. Interestingly, although a diet of *B. s.* 168 Δ*atp*(+Glu) extended lifespan more markedly than 168 Δ*atp*(-Glu), it had a less beneficial effect on thrashing and stress resistance. As reviewed in Chapter 1, the relationship between lifespan and stress resistance is complex, but these two traits are not always positively associated. A relevant example is provided by the *C. elegans* mitochondrial

complex III mutant, *isp-1,* which has increased resistance to oxidative stress as well as extended lifespan. However, a recent study reported that the longevity of this mutant does not arise from its enhanced stress response, as deletion of *sod-3* and *sod-5* compromised lifespan while increasing oxidative stress resistance (Dues *et al.*, 2017). The data presented in this chapter support the view that resistance to oxidative stress does not necessarily entail longevity, and also corroborate the results of Chapter 3 in which nematodes fed on the spore-forming *B. s.* ∆*atp* had enhanced motility without an increase in lifespan. This suggests that beneficial aspects of ATP synthase-deficient *B. subtilis* metabolism in the absence of glucose are shared between the spore-forming strain and the 168 strains used in this chapter.

In future, work should be done to extend the conclusions of this study and better define the healthspan of these animals. Improvements in motility and oxidative stress resistance in animals fed on *B. s.* 168 ∆*atp* were maintained until at least day 15 and 8, respectively, around 40-70% of their mean lifespan. The assays should be extended even further into advanced age. A simple approach might be to measure health at different timepoints encompassing a majority of maximum lifespan and quantify the percentage of animals with at least 50% of the maximal response for each health parameter (e.g. thrashing, pharyngeal pumping). These animals could be considered 'healthy', whilst those with below 50% functional capacity are frail. After normalising to maximum lifespan, the healthspan of *C. elegans* as a proportion of maximum lifespan could be compared (Sonowal *et al.*, 2017).

Such an analysis is important to determine whether health is maintained in late life. One possibility is that, although health is improved in young/middle-aged animals, healthspan is not extended. Alternatively, in the later period of their life, health might decline rapidly leading to a greater period of frailty. In the case of animals fed on *B. s.* 168 ∆*atp,* any such effect is likely to be limited, because lifespan is only marginally extended. An increased period of frailty is usually observed in situations where lifespan is extended beyond normal limits (Tissenbaum, 2012). Moreover, as previously discussed, the frequent observation of disproportionate late-life frailty in long-lived *C. elegans* mutants may simply be an artefact of raising *C. elegans* on the mildly pathogenic diet of *E. coli* (Podshivalova *et al.*, 2017). Notably, there was no indication that animals fed on a diet of *B. s.* 168

Δ*atp* were suffering from greater morbidity at later age during the lifespan assays (though this was not quantified). Such observations suggest that *B. s.* Δ*atp*(-Glu) is likely to improve healthspan without an extension of frailty; however, it will be necessary to investigate this in future.

The work presented here displays some parallels to a recently published report on the effect of bacterial indoles on healthspan in *C. elegans* (Sonowal *et al.*, 2017). Here, a diet of indole-producing *E. coli* K12, or application of indoles, consistently shifted the survival curve to the right without increasing maximal lifespan, compared to indole-deficient *E. coli* or vehicle control, respectively. However, thrashing, pharyngeal pumping, stress resistance and reproductive span was improved late in life in animals fed in the presence of indoles. Although more replicates will need to be undertaken in order for the difference in maximal lifespan to be statistically analysed, maximal lifespan of *C. elegans* fed on *B. s.* 168 Δ*atp*(-Glu) was very similar to that of 168 MS(-Glu)-fed animals (24 and 25 days, respectively). However, consistent with the effect of bacterial indoles, the lifespan curve of animals fed on 168 Δ*atp*(-Glu) was shifted to the right. Intriguingly, the same pattern of increased median, but not maximum, lifespan, was induced by the DR mimetic metformin. This involved activation of SKN-1 in the ASI neurons and intestine where it mediates DR-induced longevity and oxidative stress resistance, respectively (Onken and Driscoll, 2010). As described in Chapter 4, a diet of spore-forming *B. s.* Δ*atp*(-Glu) elicited a DR-like upregulation of *pha-4*, although the activity of SKN-1 (as judged by *Pgst-4::gfp* expression) was not increased in young adults. Such a shift in the lifespan curve may be characteristic of interventions that improve healthspan without an attendant lifespan increase. This shape seems to reflect a steeper mortality rate increase following a period of enhanced health.

### 6.5.5 *B. s.* 168 Δ*atp* stimulated DAF-16::GFP re-localisation

When *C. elegans* were fed on *B. s.* 168 Δ*atp,* DAF-16::GFP localisation to the nucleus was increased. Like motility and resistance to paraquat, nuclear localisation was slightly more noticeable in the absence of glucose. The expression of DAF-16 isoforms A/B were monitored using this reporter strain – DAF-16A in particular has an important role in transducing DAF-2/IGFR signalling in *C. elegans* (A. T.-Y. Chen *et al.*, 2015). A diet of ATP synthase-deficient *B. subtilis* therefore potentially works through a pathway involving IIS signalling to improve health and stress resistance. It should be noted that the strain used here was subsequently

found to contain a transversion mutation that results in an amino acid change in the DAF-16A sequence, although the encoded GFP fusion protein is able to rescue *daf-16* null animals (Henderson and Johnson, 2001). It would be informative to quantify localisation of DAF-16F, which is transcribed from a different promoter and also promotes longevity upon suppression of IIS (A. T.-Y. Chen *et al.*, 2015). Although this requires further investigation, the qRT-PCR analysis in Chapter 4 indicates that the expression of *daf-16* is elevated in animals fed on *B. s.* Δ*atp*(-Glu). Importantly, DR downregulates signalling through IIS to exert at least a component of its effects on longevity (Fontana *et al.*, 2010), so activation of DAF-16 could in part reflect DR.

Not all interventions that work through DAF-16, however, stimulate entry of the transcription factor into the nucleus. For example, solid DR was not able to extend *C. elegans* lifespan in *daf-16* mutants yet did not stimulate DAF-16 nuclear localisation (Greer *et al.*, 2007). Instead, AMPK activates DAF-16, possibly by direct phosphorylation, and the two factors operate in a positive feedback loop to accelerate DAF-16 target gene expression (Tullet *et al.*, 2014). In future, the involvement of DAF-16 and insulin-signalling through DAF-2 should be clarified by measuring the lifespan and health of *daf-16(mu86)* (null) mutants fed on the *B. subtilis* diets.

### 6.5.6 Matrix formation as a confounding factor

Individuals fed on 168 Δ*atp*(+Glu) were longer-lived than those fed on 168 MS(+Glu). The proportion of nematodes fed on 168 Δ*atp*(+Glu) found outside of food was high on day 2 of adulthood (around one quarter of the population). This suggests that animals fed on 168 Δ*atp*(+Glu) experienced food shortage problems similar to those of animals fed on 168 Δ*atp*(-Glu) (which were alleviated by the addition of more food), but here as a result of glucose-stimulated growth rather than inadequate growth. However, when *C. elegans* were fed on the spore-forming diets (Chapter 4), the proportion of the population outside of bacteria (zone 4) was not clearly correlated with average lifespan, and it was concluded that food avoidance was not likely to have been necessary for longevity. Indeed, the behaviour of animals fed on spore-forming Δ*atp*(+Glu) and MS(+Glu) was roughly similar, with a slight bias towards zone 3 growth in animals fed on the mutant diet (Chapter 4). In the current chapter, *C. elegans* was only found in zone 4 when they were fed on *B. s.* 168 Δ*atp* (with a substantially higher sample size than previously). Whether dietary restriction reflected in increased roaming was in fact related to the longevity

of animals fed on ATP synthase-deficient *B. subtilis* is therefore still a matter of debate. Interestingly, roaming on plates seeded with the spore-less 168 Δ*atp*(+Glu) was if anything higher than was previously observed with spore-forming *B. s.* Δ*atp*(+Glu) in spite of the matrix taking longer to develop. The extent to which roaming reflected feeding obstruction as a result of matrix deposition on these plates is therefore unclear. As discussed previously, it will be necessary to conduct a higher resolution analysis of nematode behaviour – in particular during early adulthood when feeding is greatest – to address these uncertainties. One key unknown is whether the quality and accessibility of food in areas of thinner (zone 3) growth is sufficient. As described in Chapter 4, certain measures should be taken to disentangle physical lawn growth from metabolic effects in the presence of glucose, and the implementation of such approaches should prove more facile using the *B. subtilis* 168 strains developed here.

There were clearly features specific to the previous spore-forming ATP synthase mutant which, amongst other things, dramatically reduced body size and delayed development when glucose was present. In fact, the addition of glucose seems to be an unnecessary complicating factor when the spore-less 168 strains are considered, except perhaps for the interesting and consistent failure of glucose to compromise lifespan or health. The results in this and previous chapters indicate that health is more positively affected without glucose, and therefore provide impetus to investigate the effects of the mutant diet specifically without this sugar.

### 6.5.7 Hypotheses to explain the probiotic effects of *B. subtilis* lacking ATP synthase

The evidence collated in this work indicate that a diet of *B. s.* lacking ATP synthase has probiotic effects on *C. elegans,* even if lifespan is not extended. Here, the potential mechanisms whereby ATP synthase-deficient *B. subtilis* might improve nematode health in the absence of glucose are considered. Broadly, these fall into two categories. Either the mechanism is passive, depending on differences in nutritional content or food intake; or, there is an active system of influence involving a specific bacterial activity that regulates ageing- or health-associated processes in *C. elegans.* These explanations are either growth-dependent or growth-independent, or a combination of the two.

**Growth-dependent explanations.** As discussed, one way in which slow growth of *B. subtilis* lacking ATP synthase could benefit *C. elegans* is through DR. However, even in the absence of DR, slower bacterial growth has been suggested to be beneficial for *C. elegans.* In one study, *C. elegans* preferred bacterial species with higher respiration and growth rates to those that were less active (Yu *et al.*, 2015), despite the fact that lifespan was reduced by more active strains. It has been suggested that more active bacteria compete for resources and metabolites including $O_2$ and D-lactic acid in the nematode intestine (Yu *et al.*, 2015), and that this might underlie the benefits afforded by respiration-deficient *E. coli* (Gomez *et al.*, 2012). An alternative explanation links growth rate to pathogenicity. *E. coli* pathogenicity is limited by reducing growth rate, and this potentially arises as a consequence of reduced colonisation of the *C. elegans* intestine (Gomez *et al.*, 2012).

It is, however, unclear whether slow growth of *E. coli per se* increases *C. elegans* lifespan (through reduced gut colonisation), or whether some specific changes in bacterial metabolism are responsible. Slowing the growth rate could instead reduce a specific pro-ageing *E. coli* activity – for example, virulence linked to folate metabolism in *E. coli* (Virk *et al.*, 2016). In support of this, the association between slowed bacterial growth and longer lifespan is not absolute. In a screen of 1000+ *E. coli* mutants, many mutations slowed bacterial growth without extending *C. elegans* lifespan (Virk *et al.*, 2016). Nevertheless, *B. subtilis* is non-pathogenic, and its beneficial effects are mediated at least in part by the release of factors like NO and CSF (Donato *et al.*, 2017). Therefore, it is perhaps unlikely that slow growth of respiration-deficient *B. subtilis*, or the reduced activity of a detrimental pathway in this strain, is responsible for improving *C. elegans* health.

A further demonstration of this is that increasing the rate of master strain growth and proliferation by adding glucose did not compromise the lifespan or health of *C. elegans*. Similarly, other studies have reported that the lifespan of *C. elegans* is actually reduced by around 14% when fed on dead as opposed to live *B. subtilis* spores (Donato *et al.*, 2017), or is unaffected by growth on live versus dead vegetative *B. subtilis* cells (Sánchez-Blanco *et al.*, 2016), indicating the absence of pathogenicity. Still, in case strain differences play a role, it will be useful to compare the lifespan of *C. elegans* on dead versus live *B. subtilis* 168.

**Growth-independent explanations.** One growth-independent mechanism that could contribute to the benefits afforded by *B. s.* Δ*atp* is a change in nutrient composition. A shift in the composition of macronutrients, like bulk protein, amino acids and carbohydrate, can influence body size (So *et al.*, 2011) and potentially induce DR independently of food intake. Notably, the effects of DR in *Drosophila* can be counteracted simply by altering the availability of essential amino acids (Grandison *et al.*, 2009). Differences in the macronutrient profiles of *B. s.* MS and Δ*atp* have not been assessed, but it will be important to undertake this in future. Alternatively, the production of micronutrients, such as iron or vitamins (MacNeil *et al.*, 2013; Watson *et al.*, 2014), may differ in the respiration mutant. In the case of micronutrients, either nutritional differences between strains or an active system of benefit/antagonism (e.g. vitamin B12 production by *Comamonas*) could elicit differences in lifespan and health. Similarly, specific changes in the metabolism of ATP synthase-deficient *B. subtilis* including the production of fermentation products could prove beneficial to *C. elegans.* These were discussed in Chapter 3.

There is some evidence that PHA-4 and TOR-signalling DR pathways are activated in nematodes fed on ATP synthase-deficient *B. subtilis*, without a reduction in feeding (Chapter 4). Whether this is true of animals fed on spore-less 168 *B. s.* Δ*atp* remains to be determined. DR-like states have been induced in *C. elegans* by growth and nutrition-independent mechanisms as discussed in Chapter 4. Namely, the production of a DR mimetic molecule by the mutant, or the lack of a diffusible signal/intestinal interaction from the mutant diet could elicit DR responses in *C. elegans.*

In another view, the benefits of an ATP synthase-deficient *B. subtilis* diet might arise through changes in REDOX homeostasis in *C. elegans.* ROS signalling appears to play a central role in the detrimental effects of *E. coli* in comparison to *B. subtilis.* The production of the antioxidant coQ by *E. coli* persistently lowers the level of ROS in *C. elegans,* compromising REDOX homeostasis in the nematode and, it is suggested, reducing lifespan (Sánchez-Blanco *et al.*, 2016). *C. elegans* fed on *B. subtilis* had 40% higher levels of cellular ROS and live longer. While the antioxidant NAC had no effect on the lifespan of *E. coli*-fed nematodes, it reduced the lifespan of animals fed on *B. subtilis* by around 30% (Sánchez-Blanco *et al.*, 2016). Interestingly, mild inhibition of mitochondrial respiration extends lifespan in diverse species, from yeast to mice (Lee *et al.*, 2010; Liu *et al.*, 2005). Inhibiting respiration

by targeting components of the respiratory chain and subunits of ATP synthase in *C. elegans* activates hypoxia-inducible factor 1-(*hif-1)* dependent gene expression (Lee *et al.*, 2010). A rise in ROS levels in *C. elegans* respiration mutants is responsible for activating HIF-1, which extends nematode lifespan. It has been speculated that the longevity of *C. elegans* fed on *B. subtilis,* which produces more ROS than *E. coli,* is partially dependent on HIF-1 (Sánchez-Blanco *et al.*, 2016).

ATP synthase mutants of *E. coli* have increased production of ROS, specifically, $O_2$ - and $H_2O_2$, in glucose minimal medium (Brynildsen *et al.*, 2013). It seems, therefore, that ROS levels are elevated in bacterial mutants of ATP synthase. This raises the question of whether differences in the oxidation state of *C. elegans* fed on ATP synthase-deficient *B. subtilis* versus MS might explain the probiotic effect of the mutant. In this view, higher ROS production by the mutant might shift the REDOX homeostasis of *C. elegans* into an even more beneficial state, enhancing the already positive effects of *B. subtilis.* Alternatively, the benefits could arise through a hormetic activation of stress response pathways mediated by, for example HIF-1 (or some combination of the two mechanisms). Both of these explanations are brought into question by the results of the stress reporter screen (Chapter 3). Antioxidant defence mechanisms were less active in *C. elegans* fed on the ATP synthase mutant, both with and without glucose, suggesting minimal ROS insult. Still, measurement of nematode stress responses in larvae fed on respiratory-deficient *B. subtilis* would be informative given the hormetic influence of larval $UPR^{mt}$ activation on lifespan in some reports (Tauffenberger *et al.*, 2016). Moreover, other stress-responsive genes might be active in these animals, so these ideas demand further investigation.

### 6.5.8 Future directions

How can these explanations be distinguished? Variability in food levels throughout the experiment, with the potential induction of DR through food shortage, is a confounding factor in the absence of glucose. As described, the $OD_{600}$ of *B. s.* 168 MS and 168 Δ*atp* cultures was equalised to 8 prior to seeding, so that the same number of cells were provided. However, their different growth rates quickly lead to large differences in food density. The bacteria were previously concentrated to avoid this issue, but this induced mitochondrial stress responses in *C. elegans.* In light of the finding that ATP synthase-deficient *B. subtilis* improved nematode health, it will be worth optimising food provision so that food is not limiting whilst minimising such effects on stress responses. Ideally, this would entail the use of

metabolically-active but growth arrested bacteria, for example through treatment with carbenicillin (Lenaerts *et al.*, 2008). Provision of concentrated lawns of these arrested diets would eliminate differences in growth rate as a potential contributor to health enhancement by *B. s.* Δ*atp*(-Glu) and could instead implicate shifts in metabolite production as causal to the probiotic effects of this diet. As well as this, the dependency of health improvement/longevity on high food concentrations could be determined as an indication of the role of DR. Usually, reducing food concentrations from around $10^{11}$ colony forming units (cfu) /ml to around $10^{8}$ cfu/ml results in a gradual increase in lifespan (Zhao *et al.*, 2013), whilst further reductions reduce lifespan through starvation. If the mutant diet is able to improve the health of *C. elegans* even when provided in high concentration (and pharyngeal pumping was unaffected), this would argue clearly against bacterial quantity-dependent effects. Moreover, killed, metabolically-inactive lawns of each diet could help to distinguish between a probiotic effect of the mutant diet and a detrimental effect of 168 MS. First, it would be useful to determine whether animals raised until L4 on 168 MS(-Glu) and then transferred onto 168 Δ*atp*(-Glu) are still healthier than animals fed on 168 MS(-Glu) throughout life. If so, animals could be transferred from live 168 MS(-Glu) (to enable development) onto UV- or antibiotic-killed lawns of 168 MS(-Glu) or 168 Δ*atp*(-Glu). In this scenario, if 168 Δ*atp*(-Glu) still improves health compared to 168 MS, this would indicate that a passive nutritional effect, or possibly the action of a secreted metabolite present in the mutant lawn, is responsible. It will be important to measure parameters including fecundity to ensure that the dead lawns are not eliciting DR in *C. elegans.*

To help to distinguish between passive and active explanations for the effects of *B. s.* Δ*atp* in this metabolically-arrested system, it would be informative to conduct a bacterial mixing experiment, in which the MS and ATP synthase mutant are mixed in different ratios based on cfu counts. If small amounts of mutant elicit enhanced health and stress resistance, this would argue for an active mode of bacterial influence (MacNeil *et al.*, 2013; Samuel *et al.*, 2016). Suggestions for future work are given in Table 6. 3.

**Table 6. 3 Hypotheses (grey shaded) and questions for future work regarding the probiotic effects of ATP synthase-deficient *B. subtilis* in the absence of glucose, with suggested experiments.**

| | |
|---|---|
| *B. s.* 168 Δ*atp* elicits DR in *C. elegans* | |
| **Question** | **Experiment(s)** |
| Do *C. elegans* fed on *B. s.* 168 Δ*atp* have morphological and physiological characteristics of DR? | Measure fecundity; Nile Red stain for fat content; Use *Plgg-1::gfp* reporter to quantify autophagy; Measure DR-related autofluorescence signatures. |
| Are DR pathways activated by *B. s.* 168 Δ*atp* (as with *B. s.* Δ*atp*)? | RNAseq analysis of *C. elegans* fed on *B. s.* 168 MS or 168 Δ*atp.* |
| Which ageing pathways are necessary for enhanced health?<br>What is the role of IIS in the effects of *B. s.* Δ*atp*? | Measure health of mutants or RNAi knockdowns for candidate genes identified following RNAseq, as well as genes in canonical ageing pathways including dietary restriction, IIS, mitochondrial response and germline signalling.<br>For IIS, determine the health of *daf-16(mu86)* and *daf-2(e1370)* animals. |
| *B. s.* 168 Δ*atp* promotes extended healthspan into very old age | |
| How does *B. s.* 168 Δ*atp* affect the ageing process in *C. elegans*? | Expand health assays to include a range of oxidants as well as resistance to biotic stress in old nematodes (day 10 >).<br>Quantify intestinal integrity and barrier function with age using a dye-feeding assay.<br>Mitochondrial morphology and fragmentation with age can also be qualitatively analysed and is a phenotype relevant to human age-related decline (Hahm *et al.*, 2015). Measure the survival and disease progression of *C. elegans* disease models including *glp-1(ar202) (*germline tumours) and strains expressing human Amyloid-β (Aβ). |

| | |
|---|---|
| Enhanced health of animals fed *B. s.* 168 Δ*atp* is dependent on low food quantity | |
| Does increasing food density abrogate health benefits? | Standardise growth and food quantity (cfu/ml) using metabolically-active but growth-arrested lawns |
| Are the benefits of 168 Δ*atp* growth-dependent? | |
| Are *C. elegans* consuming *B. s.* 168 Δ*atp*? | Use *B. s.* 168 strains expressing plasmid phymKATE (an *amyE* integration plasmid with constitutive mKATE expression driven by a Phyper-spank promoter), generated as part of this project. |
| Is *B. s.* 168 Δ*atp* efficiently digested? | Quantify incorporation of 168 Δ*atp* into newly-synthesised proteins using a pulse-feeding assay (Gomez-Amaro *et al.*, 2015). |
| Enhanced health of animals fed *B. s.* 168 Δ*atp* is dependent on shifts in nutrient composition | |
| Does 168 Δ*atp* have altered macronutrient or micronutrient composition? | Measure bulk levels of protein, carbohydrate and fats.<br>Measure the health of *C. elegans* on UV/heat-killed lawns of *B. s.* 168 MS and 168 Δ*atp*. |
| Differences in health arise from changes in the production of bacterial metabolites | |
| Do 168 MS and 168 Δ*atp* produce detrimental or beneficial metabolites, respectively, to influence nematode health? | Perform a media-swap experiment: resuspend UV-treated *E. coli* or 168 MS cells in spent media from 168 Δ*atp,* and vice versa, and determine health of *C. elegans.*<br>Untargeted and targeted (e.g. NO, fermentation products, $H_2S$, thiamine) metabolomics (HPLC-MS) of *B. s.* 168 MS or 168 Δ*atp*<br>Testing of purified candidate factors on lawns of metabolically-arrested or dead *B. s.* 168 MS or *E. coli* OP50.<br>Knockout production / prevent accumulation of candidate metabolites released by *B. subtilis* 168. |
| | |

| | |
|---|---|
| Gut colonisation is important for the probiotic effects of *B. s.* 168 Δ*atp* | |
| Does 168 Δ*atp* persist in the nematode intestine? | Measure *C. elegans* coliform counts (cfu/nematode) following feeding on the *B. s.* 168 diets over time (Gomez *et al.*, 2012). Quantify defecation using fluorescent mKATE strains |
| Enhanced stress resistance is independent of stress-induced hormesis | |
| Are stress responses activated by *B. s.* 168 Δ*atp* in the absence of other external stressors?<br>If so, is stress response activation necessary for health benefits? | Confirm low activation of UPRmt and cytosolic oxidative stress responses, and investigate activation of the heat shock response (*Phsp-16::gfp*) and endoplasmic reticulum UPR (*Phsp-4::gfp*).<br>Target these components using mutants or RNAi. |
| Do changes in the oxidation state of *C. elegans* on each diet underlie the health benefits of *B. s.* 168 Δ*atp*? | Measure ROS production in *C. elegans* fed on each diet.<br>Measure health following the application of antioxidants including N-acetylcysteine (NAC). |
| Is feeding on *B. s.* 168 Δ*atp* throughout larval development necessary or sufficient for health benefits? | Develop *C. elegans* on *B. s.* MS(-Glu) until L4 and subsequently culture on the mutant lawn (+phleomycin to inhibit 168 MS growth) (and vice versa). |

This thesis has focused on a single respiratory-deficient mutant of *B. subtilis.* However, it provides impetus to explore other similar mutants of electron transport and oxidative phosphorylation. Many respiration genes are essential in *B. subtilis* and lead to lethality when mutated (Kobayashi *et al.*, 2003). However, no cytochrome structural genes, and only three cytochrome biogenesis genes, are essential. One potential target is *resD,* which is responsible for the expression of genes required for heme A synthesis, and those encoding for components of the cytochrome *bf* complex (Sun *et al.*, 1996). *resD* mutants cannot grow anaerobically on nitrate and accumulate acids in the presence of glucose. Interestingly, the signal for oxygen deficiency and stimulation of biofilm formation via KinB in plant root-colonising *Bacillus amyloliquefaciens* is sensed by ResD and ResE, again linking mutation of respiratory components in *Bacillus* with biofilm formation (Zhou *et al.*, 2018).

### 6.5.9 Conclusions

It has become clear that certain benefits afforded by ATP synthase-deficient *B. subtilis* in the absence of glucose are greater than in its presence. Although lifespan is longer with glucose present, the mutant diet elicits a striking enhancement of oxidative stress resistance, as well as a more marked maintenance of muscle function with age (prolonged pharyngeal pumping and thrashing) in the absence of additional sugar. This uncoupling of lifespan from health and stress resistance offers an opportunity to uncover the molecular determinants of sustained health, which are currently more mysterious than those influencing longevity. The data presented in this chapter also highlights a potential role for DR, this time as a consequence of insufficient growth in the absence of glucose rather than lawn overgrowth in its presence. Whether the upregulation of DR pathways in nematodes fed on *B. s.* Δ*atp*(-Glu) results from passive food shortage or active modulation of signalling by *B. subtilis* metabolites remains, therefore, to be determined. Overall, the 168 strains are easier to work with than *B. s.* MS and Δ*atp.* The formation of a matrix by *B. s.* 168 Δ*atp* is slowed, but not eliminated, enabling further investigation into its effects whilst minimising the confounding effects of its overgrowth. Spore formation is removed as a variable in the experiments, and genetic manipulation of these strains is facile. Future work should focus on determining the contribution of DR to the enhanced health of *C. elegans* fed on *B. s.* 168 Δ*atp,* as well as standardising the cell number by implementing the suggestions above.

# 7 General discussion and conclusions

## 7.1 Summary of findings

The use of a diverse range of bacterial diets has highlighted that the interactions between *C. elegans* and its dietary microbes are dynamic and context-dependent. Along with changes in microbial composition, bacterial metabolic mutants in pathways regulating the synthesis of folate (Virk *et al.*, 2016), methionine (Cabreiro *et al.*, 2013) and colonic acid (Han *et al.*, 2017), as well as defects in bacterial respiratory metabolism (Gomez *et al.*, 2012; Saiki *et al.*, 2008), can profoundly influence host ageing. Genetic heterogeneity within microbial populations therefore tunes host longevity and health (Han *et al.*, 2017).

Here, the influence of a respiratory-deficient *Bacillus subtilis* mutant on lifespan, health and stress responses in *C. elegans* was investigated. While investigation into the effects of glucose in *C. elegans* has primarily focused on its direct toxicity, the focus of this thesis is on the indirect effect of glucose in the context of the microbiota. The ATP synthase mutant was observed to extend the lifespan of *C. elegans* in the presence of glucose, but not (or to a lesser extent) in its absence. This diet also stimulated entry into L1 arrest, reduced adult body size, delayed egg laying, increased motility and activated the expression of genes associated with DR in the nematode when glucose was present. The additivity of this diet with mutation of *eat-2(ad1116)* leaves open the possibility that other pro-longevity mechanisms might be working in parallel with DR. The evidence currently points to a role for feeding obstruction by the overgrown extracellular matrix on lawns of the mutant diet, although it is still possible that active host-microbiome signalling is involved. Specifically, the work in Chapter 6 in which matrix formation is limited and sporulation is prevented points to there being a component of longevity which is distinct from these factors. To make full use of the nematode-microbe system, the influence of gut microbiota on host physiology through their effect on cells and tissues must be further investigated. Currently, microbe-mediated germ cell proliferation (Chaudhari *et al.*, 2016), mitochondrial fragmentation (Han *et al.*, 2017) and age-related invasion of the pharynx and intestine (Ezcurra, 2018; Podshivalova *et al.*, 2017; Y. Zhao *et al.*, 2017) are examples of such interactions in this system.

Unexpectedly, the work in this project has demonstrated that ATP synthase-deficient *B. subtilis* is beneficial even, and perhaps especially, without glucose. Evidence is presented that compromising oxidative phosphorylation through mutation of a deeply conserved enzyme in a natural diet of *C. elegans* enhances the already beneficial nature of this diet, and disproportionately affects health as opposed to lifespan. These effects are not dependent on the presence of spores and appear to be unrelated to adult activation of hormetic mitochondrial and cytosolic stress responses. The observation that thrashing motility and pharyngeal pumping frequency were maintained at older ages in these individuals reveals a sustainment of muscular and neurological function and indicates that healthspan was improved by this diet. In the spore-less parental 168 strain developed in Chapter 6, resistance to oxidative stress was markedly enhanced by the mutant even into mid-adulthood, and glucose largely compromised this stress resistance.

In the original spore-forming background, *B. s.* Δ*atp*(-Glu) appeared to be eliciting DR, as in the presence of glucose. This was based primarily on a candidate qRT-PCR screen of DR regulators – although a delay in development and reduced fecundity are also consistent with DR. The lack of overlap in the physiology of animals fed on the mutant diet in the absence and the presence of glucose suggests, however, that DR is working through distinct pathways in each case. This is perhaps to be expected, as the overgrowth of an extracellular matrix in the presence but not in the absence of glucose appears to be critical. Extensive spore formation by *B. s.* Δ*atp*(-Glu), as well as the possibility of insufficient food quantity, are highlighted as potential mechanisms underlying these effects. Whether similar DR pathways were elicited by the spore-less ATP synthase mutant, or whether they were activated specifically in response to an abundance of spores, is an important question that remains unanswered. The observation that both spore-forming and spore-less diets of the mutant disproportionately improved health over lifespan shows that there is likely to be a shared response to each diet operating through similar pathways. The spore-less 168 strain will facilitate investigation into the nature of these pathways. Potential mechanisms range from altered conditioning of the nematode intestine to the release of pro-longevity or pro-health metabolites that activate anti-ageing pathways in the nematode. Microbiome-derived signals and activities have been shown to interact with conserved longevity pathways including mTOR, HSF-1 and DAF-16/FOXO, to influence lifespan, health and stress responses in *C. elegans* (Donato *et al.*, 2017). Unbiased metabolomic and

transcriptomic analyses are being initiated in order to expand the range of targets and better define the response of *C. elegans* to this diet.

### 7.2 Implications for the study of host-microbiota interactions

The potential future impact of these findings is wide-ranging. Various considerations related to bacterial growth, metabolism and resultant changes in physico-chemical lawn properties are highlighted, with practical implications for the use of non-standard nematode diets. The approach taken, of increasing standardisation of growth conditions, minimisation of confounding factors through changes in strain background, and progress in the construction of RNAi-capable *B. subtilis* strains, represent important steps in the development of this system for future investigation, and should inform future forays into alternative nematode diets. The interaction between *C. elegans* and its food source reflected in the roaming-induced growth of zone 3 bacteria on NGM(+Glu) plates, and the preferential dwelling in these regions instead of matrix-overgrown areas, draws attention to the dynamic nature of *C. elegans* feeding and food searching. Similar behaviours in response to heterogenous conditions might be relevant in the natural habitat of the nematode. In the context of research using *E. coli*, the finding that ATP synthase-deficient *B. subtilis* is able to benefit *C. elegans* implies that the positive effects of slow-growing respiratory-deficient *E. coli* strains might have an actively probiotic component, rather than simply reflecting reduced bacterial pathogenicity. In support of this, the lifespan of *C. elegans* fed on coQ-less *E. coli* was reduced by blocking bacterial proliferation with UV exposure (Saiki *et al.*, 2008). Moreover, there is a clear implication from these findings that the physiology of *C. elegans* in the presence of different carbon sources can be dramatically altered by the composition of its microbiota. This provides impetus to define how other members of the natural commensal consortia of *C. elegans* mediate the effects of dietary constituents, which fits well into the increasing interest in and understanding of the core microbial community of the nematode (Shapira, 2017).

Furthermore, these findings potentially relate to interactions between the mammalian intestine and the resident microbiota. Studies have convincingly linked the composition and activity of the microbiota to ageing in humans. Relatedly, dietary components and microbial dysbiosis have been associated with various inflammatory diseases, and alteration of the microbial community has been pursued

as an avenue for limiting pathology and augmenting damage resistance (Richards *et al.*, 2016). Model organisms offer an opportunity to understand these complex relationships, and the relevance of *C. elegans* as a system for such aims is beyond doubt; activation of classical longevity pathways and delayed ageing have been elicited by feeding the nematode on bacterial strains isolated from the intestine of humans, including centenarians (Ezcurra, 2018; Nakagawa *et al.*, 2016; L. Zhao *et al.*, 2017). Application of such findings to mammalian systems is facilitated by the suitability of *B. subtilis* for probiotic interventions (Permpoonpattana *et al.*, 2012). There is good precedent for the utility of this species in a mammalian context; *B. subtilis* is widely used as an alternative to antibiotics in animal feed (Cutting, 2011), improves gut-associated lymphoid tissue (Huang *et al.*, 2008) and competitively excludes enteropathogens by adhering to intestinal cells (La Ragione and Woodward, 2003; Poormontaseri *et al.*, 2017; Ye *et al.*, 2013). Several commercialised probiotic health supplements containing *B. subtilis* strains exist for human consumption**,** including Biosporin (Europe) and Primal Defense (USA). Manipulation of the respiratory metabolism of *B. subtilis* is presented here as a potential approach for enhancing the probiotic capabilities of this diet in mammalian systems.

Dietary glucose is associated with a range of chronic diseases in humans, and these problems are becoming exacerbated in an ageing population. Moreover, changes in bacterial metabolism stimulated by shifts in glucose availability can influence processes such as immune system function in humans. For example, the differentiation of T helper 17 cells is stimulated by glucose-dependent ATP secretion from commensal gut bacteria including *Enterococcus* species (Hironaka *et al.*, 2013). Within this context, the work in this thesis focused largely on the ability of a respiratory-deficient mutant of *B. subtilis* to extend the lifespan of *C. elegans* in the presence of dietary glucose. Whilst glucose is usually toxic to *C. elegans,* its presence did not affect or extended nematode lifespan in the presence of *B. subtilis.* These findings offer impetus for further research into how exactly *B. subtilis* metabolism mediates the effects of this sugar on the host, and whether such effects are generalisable to other members of *Bacilli* including the probiotic commensal *B. megaterium* (Coolon *et al.*, 2009; Montalvo-Katz *et al.*, 2013) and *B. subtilis* GS67 (Iatsenko *et al.*, 2014). Heightened production of extracellular matrix by respiratory-deficient *B. subtilis* is highlighted as a potential mechanism that interacts with glucose availability to delay ageing in the nematode. If similar mechanisms operate in the human gut, possible targets for modulating the toxicity of glucose and

intervening in human ageing and chronic disease may arise following the identification of molecular and physiological effectors.

Additionally, by considering health and stress resistance as well as lifespan, a marked benefit of ATP synthase-deficient *B. subtilis* grown without high levels of exogenous glucose was uncovered, reflected in improved health without longevity. Further investigation into this system is a promising route for understanding how the microbiota can uncouple healthspan from lifespan, which can in turn provide valuable insight into the currently mysterious processes that regulate health independently from longevity. Ultimately, such knowledge is of significant relevance for the medical goal of promoting healthy ageing and compressing the period of frailty in human populations.

**Bibliography**

**Abada, E. A.-e., Sung, H., Dwivedi, M., Park, B.-J., Lee, S.-K., and Ahnn, J.** (2009). *C. elegans* behavior of preference choice on bacterial food. *Mol. Cells,* **28**(3), 209-213.

**Aitlhadj, L., and Sturzenbaum, S. R.** (2010). The use of FUdR can cause prolonged longevity in mutant nematodes. *Mech. Ageing Dev.,* **131**(5), 364-365.

**Altun, Z., and Hall, D.** (2018). Handbook of *C. elegans* Anatomy. In *WormAtlas*. doi:10.3908/wormatlas.1.1

**Anderson, E. N., Corkins, M. E., Li, J.-C., Singh, K., Parsons, S., Tucey, T. M., Sorkaç, A., Huang, H., Dimitriadi, M., Sinclair, D. A., and Hart, A. C.** (2016). *C. elegans* lifespan extension by osmotic stress requires FUdR, base excision repair, FOXO, and sirtuins. *Mech. Ageing Dev.,* **154**, 30-42.

**Apfeld, J., O'Connor, G., McDonagh, T., DiStefano, P. S., and Curtis, R.** (2004). The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in *C. elegans*. *Genes Dev.,* **18**(24), 3004-3009.

**Aston, L. M.** (2006). Glycaemic index and metabolic disease risk. *Proc. Nutr. Soc.,* **65**(01), 125-134.

**Avery, L.** (1993). The genetics of feeding in *Caenorhabditis elegans*. *Genetics,* **133**(4), 897-917.

**Avery, L., and Horvitz, H. R.** (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron,* **3**(4), 473-485.

**Avery, L., and Shtonda, B. B.** (2003). Food transport in the *C. elegans* pharynx. *J. Exp. Biol.,* **206**(14), 2441-2457.

**Ayyadevara, S., Dandapat, A., Singh, S. P., Beneš, H., Zimniak, L., Reis, R. J. S., and Zimniak, P.** (2005). Lifespan extension in hypomorphic *daf-2* mutants of *Caenorhabditis elegans* is partially mediated by glutathione transferase CeGSTP2-2. *Aging cell,* **4**(6), 299-307.

**Bader, J., Albin, A., and Stahl, U.** (2012). Spore-forming bacteria and their utilisation as probiotics. *Beneficial microbes,* **3**(1), 67-75.

**Bansal, A., Zhu, L. J., Yen, K., and Tissenbaum, H. A.** (2015). Uncoupling lifespan and healthspan in *Caenorhabditis elegans* longevity mutants. *Proc. Natl. Acad. Sci. U.S.A.,* **112**(3), E277-E286.

**Bar, D. Z., Charar, C., Dorfman, J., Yadid, T., Tafforeau, L., Lafontaine, D. L., and Gruenbaum, Y.** (2016). Cell size and fat content of dietary-restricted *Caenorhabditis elegans* are regulated by ATX-2, an mTOR repressor. *Proc. Natl. Acad. Sci. U.S.A.,* **113**(32), E4620-E4629.

**Baugh, L. R.** (2013). To Grow or Not to Grow: Nutritional Control of Development During *Caenorhabditis elegans* L1 Arrest. *Genetics,* **194**(3), 539-555.

**Ben-Yehuda, S., Rudner, D. Z., and Losick, R.** (2003). RacA, a bacterial protein that anchors chromosomes to the cell poles. *Science,* **299**(5606), 532-536.

**Bennett, C. F., and Kaeberlein, M.** (2014). The mitochondrial unfolded protein response and increased longevity: cause, consequence, or correlation? *Exp. Gerontol.,* **56**, 142-146.

**Bennett, C. F., Wende, H. V., Simko, M., Klum, S., Barfield, S., Choi, H., Pineda, V. V., and Kaeberlein, M.** (2014). Activation of the mitochondrial unfolded protein response does not predict longevity in *Caenorhabditis elegans*. *Nature communications,* **5**, 3483-3483.

**Berg, M., Stenuit, B., Ho, J., Wang, A., Parke, C., Knight, M., Alvarez-Cohen, L., and Shapira, M.** (2016). Assembly of the *Caenorhabditis elegans* gut microbiota from diverse soil microbial environments. *The ISME journal,* **10**(8), 1998-2009.

**Bishop, N. A., and Guarente, L.** (2007). Two neurons mediate diet-restriction-induced longevity in *C. elegans*. *Nature,* **447**(7144), 545-549.

**Blagosklonny, M. V.** (2012). Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging. *Aging (Albany NY),* **4**(3), 159-165.

**Brenner, S.** (1974). The genetics of *Caenorhabditis elegans*. *Genetics,* **77**(1), 71-94.

**Britton, R. A., Eichenberger, P., Gonzalez-Pastor, J. E., Fawcett, P., Monson, R., Losick, R., and Grossman, A. D.** (2002). Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. *J. Bacteriol.,* **184**(17), 4881-4890.

**Brokate-Llanos, A. M., Garzón, A., and Muñoz, M. J.** (2014). *Escherichia coli* carbon source metabolism affects longevity of its predator *Caenorhabditis elegans*. *Mech. Ageing Dev.,* **141**, 22-25.

**Brooks, K. K., Liang, B., and Watts, J. L.** (2009). The Influence of Bacterial Diet on Fat Storage in C. elegans. *PLOS ONE,* **4**(10), e7545.

**Browning, D. F., Wells, T. J., França, F. L., Morris, F. C., Sevastsyanovich, Y. R., Bryant, J. A., Johnson, M. D., Lund, P. A., Cunningham, A. F., and Hobman, J. L.** (2013). Laboratory adapted *Escherichia coli* K-12 becomes a pathogen of *Caenorhabditis elegans* upon restoration of O antigen biosynthesis. *Mol. Microbiol.,* **87**(5), 939-950.

**Brynildsen, M. P., Winkler, J. A., Spina, C. S., MacDonald, I. C., and Collins, J. J.** (2013). Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production. *Nat. Biotechnol.,* **31**(2), 160.

**Cabreiro, F., Ackerman, D., Doonan, R., Araiz, C., Back, P., Papp, D., Braeckman, B. P., and Gems, D.** (2011). Increased life span from overexpression of superoxide dismutase in *Caenorhabditis elegans* is not caused by decreased oxidative damage. *Free Radical Biology and Medicine,* **51**(8), 1575-1582.

**Cabreiro, F., Au, C., Leung, K.-Y., Vergara-Irigaray, N., Cochemé, Helena M., Noori, T., Weinkove, D., Schuster, E., Greene, Nicholas D. E., and Gems, D.**

(2013). Metformin Retards Aging in *C. elegans* by Altering Microbial Folate and Methionine Metabolism. *Cell,* **153**(1), 228-239.

**Cai, H., Rasulova, M., Vandemeulebroucke, L., Meagher, L., Vlaeminck, C., Dhondt, I., and Braeckman, B. P.** (2017). Life-Span Extension by Axenic Dietary Restriction Is Independent of the Mitochondrial Unfolded Protein Response and Mitohormesis in *Caenorhabditis elegans*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* **72**(10), 1311-1318.

**Carniol, K., Ben-Yehuda, S., King, N., and Losick, R.** (2005). Genetic dissection of the sporulation protein SpoIIE and its role in asymmetric division in *Bacillus subtilis*. *J. Bacteriol.,* **187**(10), 3511-3520.

**Castelein, N., Cai, H., Rasulova, M., and Braeckman, B. P.** (2014). Lifespan regulation under axenic dietary restriction: a close look at the usual suspects. *Exp. Gerontol.,* **58**, 96-103.

**Chaudhari, S. N., Mukherjee, M., Vagasi, A. S., Bi, G., Rahman, M. M., Nguyen, C. Q., Paul, L., Selhub, J., and Kipreos, E. T.** (2016). Bacterial folates provide an exogenous signal for *C. elegans* germline stem cell proliferation. *Dev. Cell,* **38**(1), 33-46.

**Chen, A. T.-Y., Guo, C., Itani, O. A., Budaitis, B. G., Williams, T. W., Hopkins, C. E., McEachin, R. C., Pande, M., Grant, A. R., Yoshina, S., Mitani, S., and Hu, P. J.** (2015). Longevity Genes Revealed by Integrative Analysis of Isoform-Specific *daf-16*/*FoxO* Mutants of *Caenorhabditis elegans*. *Genetics,* **201**(2), 613-629.

**Chen, D., Thomas, E. L., and Kapahi, P.** (2009). HIF-1 Modulates Dietary Restriction-Mediated Lifespan Extension via IRE-1 in *Caenorhabditis elegans*. *PLoS Genetics,* **5**(5), e1000486.

**Chen, J., Ou, Y., Li, Y., Hu, S., Shao, L.-W., and Liu, Y.** (2017). Metformin extends *C. elegans* lifespan through lysosomal pathway. *eLife,* **6**, e31268.

**Chen, Y., Gozzi, K., Yan, F., and Chai, Y.** (2015). Acetic acid acts as a volatile signal to stimulate bacterial biofilm formation. *MBio,* **6**(3), e00392-00315.

**Chin, R. M., Fu, X., Pai, M. Y., Vergnes, L., Hwang, H., Deng, G., Diep, S., Lomenick, B., Meli, V. S., Monsalve, G. C., Hu, E., Whelan, S. A., Wang, J. X., Jung, G., Solis, G. M., Fazlollahi, F., Kaweeteerawat, C., Quach, A., Nili, M., Krall, A. S., Godwin, H. A., Chang, H. R., Faull, K. F., Guo, F., Jiang, M., Trauger, S. A., Saghatelian, A., Braas, D., Christofk, H. R., Clarke, C. F., Teitell, M. A., Petrascheck, M., Reue, K., Jung, M. E., Frand, A. R., and Huang, J.** (2014). The metabolite alpha-ketoglutarate extends lifespan by inhibiting the ATP synthase and TOR. *Nature,* **510**(7505), 397-401.

**Chiu, C.-J., Liu, S., Willett, W. C., Wolever, T. M. S., Brand-Miller, J. C., Barclay, A. W., and Taylor, A.** (2011). Informing food choices and health outcomes by use of the dietary glycemic index. *Nutrition reviews,* **69**(4), 231-242.

**Choi, S. S.** (2011). High glucose diets shorten lifespan of *Caenorhabditis elegans* via ectopic apoptosis induction. *Nutrition Research and Practice,* **5**(3), 214-218.

**Chow, D. K., Glenn, C. F., Johnston, J. L., Goldberg, I. G., and Wolkow, C. A.** (2006). Sarcopenia in the *Caenorhabditis elegans* pharynx correlates with muscle contraction rate over lifespan. *Exp. Gerontol.,* **41**(3), 252-260.

**Chuang, M.-H., Chiou, S.-H., Huang, C.-H., Yang, W.-B., and Wong, C.-H.** (2009). The lifespan-promoting effect of acetic acid and Reishi polysaccharide. *Bioorg. Med. Chem.,* **17**(22), 7831-7840.

**Clark, R. I., and Walker, D. W.** (2018). Role of gut microbiota in aging-related health decline: insights from invertebrate models. *Cellular and Molecular Life Sciences,* **75**(1), 93-101.

**Cohen, E., Bieschke, J., Perciavalle, R. M., Kelly, J. W., and Dillin, A.** (2006). Opposing activities protect against age-onset proteotoxicity. *Science,* **313**(5793), 1604-1610.

**Cole, L. A.** (2016). Chapter 10 - Adenosine Triphosphate Energetics. In *Biology of Life,* pp. 65-77. Edited by L. A. Cole. Academic Press.

**Coolon, J. D., Jones, K. L., Todd, T. C., Carr, B. C., and Herman, M. A.** (2009). *Caenorhabditis elegans* genomic response to soil bacteria predicts environment-specific genetic effects on life history traits. *PLoS Genet,* **5**(6), e1000503.

**Corsi, A. K., Wightman, B., and Chalfie, M.** (2015). A transparent window into biology: a primer on *Caenorhabditis elegans*. *Genetics,* **200**(2), 387-407.

**Cutting, S. M.** (2011). *Bacillus* probiotics. *Food Microbiology,* **28**(2), 214-220.

**de Jong, L., Meng, Y., Dent, J., and Hekimi, S.** (2004). Thiamine pyrophosphate biosynthesis and transport in the nematode *Caenorhabditis elegans*. *Genetics,* **168**(2), 845-854.

**Detienne, G., Van de Walle, P., De Haes, W., Schoofs, L., and Temmerman, L.** (2016). SKN-1-independent transcriptional activation of glutathione S-transferase 4 (GST-4) by EGF signaling. *Worm,* **5**(4), e1230585.

**Dirksen, P., Marsh, S. A., Braker, I., Heitland, N., Wagner, S., Nakad, R., Mader, S., Petersen, C., Kowallik, V., Rosenstiel, P., Félix, M.-A., and Schulenburg, H.** (2016). The native microbiome of the nematode *Caenorhabditis elegans*: gateway to a new host-microbiome model. *BMC Biology,* **14**, 38.

**Dixon, L. G., and Spiegelman, G. B.** (2002). Glucose-resistant sporulation in *Bacillus subtilis crsA47* mutants does not depend on promoter switching at the *spo0A* gene. *J. Bacteriol.,* **184**(5), 1458-1461.

**Donato, V., Ayala, F. R., Cogliati, S., Bauman, C., Costa, J. G., Leñini, C., and Grau, R.** (2017). *Bacillus subtilis* biofilm extends *Caenorhabditis elegans* longevity through downregulation of the insulin-like signalling pathway. *Nature Communications,* **8**, 14332.

**Doonan, R., McElwee, J. J., Matthijssens, F., Walker, G. A., Houthoofd, K., Back, P., Matscheski, A., Vanfleteren, J. R., and Gems, D.** (2008). Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. *Genes Dev.,* **22**(23), 3236-3241.

**Dorman, J. B., Albinder, B., Shroyer, T., and Kenyon, C.** (1995). The *age-1* and *daf-2* genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*. *Genetics,* **141**(4), 1399-1406.

**Dues, D. J., Schaar, C. E., Johnson, B. K., Bowman, M. J., Winn, M. E., Senchuk, M. M., and Van Raamsdonk, J. M.** (2017). Uncoupling of oxidative stress resistance and lifespan in long-lived *isp-1* mitochondrial mutants in *Caenorhabditis elegans*. *Free Radical Biology and Medicine,* **108**, 362-373.

**Durand, S., Gilet, L., and Condon, C.** (2012). The Essential Function of *B. subtilis* RNase III Is to Silence Foreign Toxin Genes. *PLOS Genetics,* **8**(12), e1003181.

**Ellis, R. E., Jacobson, D. M., and Horvitz, H. R.** (1991). Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics,* **129**(1), 79-94.

**Elshaghabee, F. M. F., Rokana, N., Gulhane, R. D., Sharma, C., and Panwar, H.** (2017). *Bacillus* As Potential Probiotics: Status, Concerns, and Future Perspectives. *Frontiers in Microbiology,* **8**, 1490.

**Ezcurra, M.** (2018). Dissecting cause and effect in host-microbiome interactions using the combined worm-bug model system. *Biogerontology*, 1-12.

**Fang-Yen, C., Avery, L., and Samuel, A. D.** (2009). Two size-selective mechanisms specifically trap bacteria-sized food particles in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.,* **106**(47), 20093-20096.

**Félix, M.-A., and Duveau, F.** (2012). Population dynamics and habitat sharing of natural populations of *Caenorhabditis elegans* and *C. briggsae*. *BMC Biology,* **10**(1), 1-19.

**Feucht, A., Magnin, T., Yudkin, M. D., and Errington, J.** (1996). Bifunctional protein required for asymmetric cell division and cell-specific transcription in *Bacillus subtilis*. *Genes Dev.,* **10**(7), 794-803.

**Fielenbach, N., and Antebi, A.** (2008). *C. elegans* dauer formation and the molecular basis of plasticity. *Genes Dev.,* **22**(16), 2149-2165.

**Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C.** (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature,* **391**(6669), 806-811.

**Fontana, L., Partridge, L., and Longo, V. D.** (2010). Extending healthy life span—from yeast to humans. *Science,* **328**(5976), 321-326.

**Frézal, L., and Félix, M.-A.** (2015). *C. elegans* outside the Petri dish. *Elife,* **4**, e05849.

**Friedman, D. B., and Johnson, T. E.** (1988). A mutation in the *age-1* gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. *Genetics,* **118**(1), 75-86.

**Fujiya, M., Musch, M. W., Nakagawa, Y., Hu, S., Alverdy, J., Kohgo, Y., Schneewind, O., Jabri, B., and Chang, E. B.** (2007). The *Bacillus subtilis* quorum-

sensing molecule CSF contributes to intestinal homeostasis via OCTN2, a host cell membrane transporter. *Cell host & microbe,* **1**(4), 299-308.

**Gallegos-Monterrosa, R., Mhatre, E., and Kovács, Á. T.** (2016). Specific *Bacillus subtilis* 168 variants form biofilms on nutrient-rich medium. *Microbiology,* **162**(11), 1922-1932.

**Garcia, A. M., Ladage, M. L., Dumesnil, D. R., Zaman, K., Shulaev, V., Azad, R. K., and Padilla, P. A.** (2015). Glucose Induces Sensitivity to Oxygen Deprivation and Modulates Insulin/IGF-1 Signaling and Lipid Biosynthesis in *Caenorhabditis elegans*. *Genetics,* **200**(1), 167-184.

**García-González, A. P., Ritter, A. D., Shrestha, S., Andersen, E. C., Yilmaz, L. S., and Walhout, A. J. M.** (2017). Bacterial Metabolism Affects the *C.elegans* Response to Cancer Chemotherapeutics. *Cell,* **169**(3), 431-441.e438.

**Garigan, D., Hsu, A.-L., Fraser, A. G., Kamath, R. S., Ahringer, J., and Kenyon, C.** (2002). Genetic analysis of tissue aging in *Caenorhabditis elegans*: a role for heat-shock factor and bacterial proliferation. *Genetics,* **161**(3), 1101-1112.

**Garsin, D. A., Sifri, C. D., Mylonakis, E., Qin, X., Singh, K. V., Murray, B. E., Calderwood, S. B., and Ausubel, F. M.** (2001). A simple model host for identifying Gram-positive virulence factors. *Proc. Natl. Acad. Sci.,* **98**(19), 10892-10897.

**Garsin, D. A., Villanueva, J. M., Begun, J., Kim, D. H., Sifri, C. D., Calderwood, S. B., Ruvkun, G., and Ausubel, F. M.** (2003). Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens. *Science,* **300**(5627), 1921-1921.

**Gelino, S., Chang, J. T., Kumsta, C., She, X., Davis, A., Nguyen, C., Panowski, S., and Hansen, M.** (2016). Intestinal Autophagy Improves Healthspan and Longevity in *C. elegans* during Dietary Restriction. *PLOS Genetics,* **12**(7), e1006135.

**Gems, D., and Partridge, L.** (2008). Stress-Response Hormesis and Aging: That which Does Not Kill Us Makes Us Stronger. *Cell Metab.,* **7**(3), 200-203.

**Gems, D., and Riddle, D. L.** (2000). Genetic, behavioral and environmental determinants of male longevity in *Caenorhabditis elegans*. *Genetics,* **154**(4), 1597-1610.

**Gerbaba, T. K., Green-Harrison, L., and Buret, A. G.** (2017). Modeling Host-Microbiome Interactions in *Caenorhabditis elegans*. *J. Nematol.,* **49**(4), 348-356.

**Gerstbrein, B., Stamatas, G., Kollias, N., and Driscoll, M.** (2005). In vivo spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in *Caenorhabditis elegans*. *Aging Cell,* **4**(3), 127-137.

**Gigliobianco, T., Gangolf, M., Lakaye, B., Pirson, B., von Ballmoos, C., Wins, P., and Bettendorff, L.** (2013). An alternative role of FoF1-ATP synthase in *Escherichia coli:* synthesis of thiamine triphosphate. *Scientific reports,* **3**, 1071.

**Gomez, F., Monsalve, G. C., Tse, V., Saiki, R., Weng, E., Lee, L., Srinivasan, C., Frand, A. R., and Clarke, C. F.** (2012). Delayed accumulation of intestinal coliform bacteria enhances life span and stress resistance in *Caenorhabditis elegans* fed respiratory deficient *E. coli*. *BMC Microbiol.,* **12**, 300-300.

**Gomez-Amaro, R. L., Valentine, E. R., Carretero, M., LeBoeuf, S. E., Rangaraju, S., Broaddus, C. D., Solis, G. M., Williamson, J. R., and Petrascheck, M.** (2015). Measuring Food Intake and Nutrient Absorption in *Caenorhabditis elegans*. *Genetics,* **200**(2), 443-454.

**Govindan, J. A., Jayamani, E., Zhang, X., Mylonakis, E., and Ruvkun, G.** (2015). Dialogue between *E. coli* free radical pathways and the mitochondria of *C. elegans*. *Proc. Natl. Acad. Sci. U.S.A.,* **112**(40), 12456-12461.

**Gracida, X., and Eckmann, C. R.** (2013). Fertility and germline stem cell maintenance under different diets requires nhr-114/HNF4 in *C. elegans*. *Curr. Biol.,* **23**(7), 607-613.

**Grandison, R. C., Piper, M. D., and Partridge, L.** (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in *Drosophila*. *Nature,* **462**(7276), 1061.

**Greer, E. L., and Brunet, A.** (2009). Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*. *Aging Cell,* **8**(2), 113-127.

**Greer, E. L., Dowlatshahi, D., Banko, M. R., Villen, J., Hoang, K., Blanchard, D., Gygi, S. P., and Brunet, A.** (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in *C. elegans*. *Curr. Biol.,* **17**(19), 1646-1656.

**Grewal, P.** (1991). Influence of bacteria and temperature on the reproduction of *Caenorhabditis elegans* (Nematoda: Rhabditidae) infesting mushrooms (Agaricus Bispor Us). *Nematologica,* **37**(1), 72-82.

**Grompone, G., Martorell, P., Llopis, S., Gonzalez, N., Genoves, S., Mulet, A. P., Fernandez-Calero, T., Tiscornia, I., Bollati-Fogolin, M., Chambaud, I., Foligne, B., Montserrat, A., and Ramon, D.** (2012). Anti-inflammatory *Lactobacillus rhamnosus* CNCM I-3690 strain protects against oxidative stress and increases lifespan in *Caenorhabditis elegans*. *PLoS One,* **7**(12), e52493.

**Gruber, J., and Kennedy, B. K.** (2017). Microbiome and Longevity: Gut Microbes Send Signals to Host Mitochondria. *Cell,* **169**(7), 1168-1169.

**Gusarov, I., Gautier, L., Smolentseva, O., Shamovsky, I., Eremina, S., Mironov, A., and Nudler, E.** (2013). Bacterial nitric oxide extends the lifespan of *C. elegans*. *Cell,* **152**(4), 818-830.

**Gusarov, I., Pani, B., Gautier, L., Smolentseva, O., Eremina, S., Shamovsky, I., Katkova-Zhukotskaya, O., Mironov, A., and Nudler, E.** (2017). Glycogen controls *Caenorhabditis elegans* lifespan and resistance to oxidative stress. *Nature Communications,* **8**, 15868.

**Hahm, J.-H., Kim, S., DiLoreto, R., Shi, C., Lee, S.-J. V., Murphy, C. T., and Nam, H. G.** (2015). *C. elegans* maximum velocity correlates with healthspan and is maintained in worms with an insulin receptor mutation. *Nature communications,* **6**, 8919.

**Han, B., Sivaramakrishnan, P., Lin, C. J., Neve, I. A. A., He, J., Tay, L. W. R., Sowa, J. N., Sizovs, A., Du, G., Wang, J., Herman, C., and Wang, M. C.** (2017). Microbial Genetic Composition Tunes Host Longevity. *Cell,* **169**(7), 1249-1262.e1213.

**Hansen, M., Chandra, A., Mitic, L. L., Onken, B., Driscoll, M., and Kenyon, C.** (2008). A Role for Autophagy in the Extension of Lifespan by Dietary Restriction in *C. elegans*. *PLOS Genetics,* **4**(2), e24.

**Hansen, M., Taubert, S., Crawford, D., Libina, N., Lee, S. J., and Kenyon, C.** (2007). Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging cell,* **6**(1), 95-110.

**Harman, D.** (1956). Aging: A Theory Based on Free Radical and Radiation Chemistry. *Journal of Gerontology,* **11**(3), 298-300.

**Heestand, B. N., Shen, Y., Liu, W., Magner, D. B., Storm, N., Meharg, C., Habermann, B., and Antebi, A.** (2013). Dietary Restriction Induced Longevity Is Mediated by Nuclear Receptor NHR-62 in *Caenorhabditis elegans*. *PLOS Genetics,* **9**(7), e1003651.

**Heintz, C., Doktor, T. K., Lanjuin, A., Escoubas, C. C., Zhang, Y., Weir, H. J., Dutta, S., Silva-García, C. G., Bruun, G. H., Morantte, I., Hoxhaj, G., Manning, B. D., Andresen, B. S., and Mair, W. B.** (2016). Splicing factor 1 modulates dietary restriction and TORC1 pathway longevity in *C. elegans*. *Nature,* **541**, 102.

**Heintz, C., and Mair, W.** (2014). You Are What You Host: Microbiome Modulation of the Aging Process. *Cell,* **156**(3), 408-411.

**Henderson, S. T., and Johnson, T. E.** (2001). *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. *Curr. Biol.,* **11**(24), 1975-1980.

**Herndon, L. A., Schmeissner, P. J., Dudaronek, J. M., Brown, P. A., Listner, K. M., Sakano, Y., Paupard, M. C., Hall, D. H., and Driscoll, M.** (2002). Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. *Nature,* **419**(6909), 808-814.

**Higashibata, A., Szewczyk, N. J., Conley, C. A., Imamizo-Sato, M., Higashitani, A., and Ishioka, N.** (2006). Decreased expression of myogenic transcription factors and myosin heavy chains in *Caenorhabditis elegans* muscles developed during spaceflight. *J. Exp. Biol.,* **209**(16), 3209-3218.

**Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., and Sanders, M. E.** (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology,* **11**, 506-515.

**Hironaka, I., Iwase, T., Sugimoto, S., Okuda, K.-i., Tajima, A., Yanaga, K., and Mizunoe, Y.** (2013). Glucose triggers ATP secretion from bacteria in a growth phase-dependent manner. *Appl. Environ. Microbiol.,* **79**(7), 2328-2335.

**Honda, Y., Tanaka, M., and Honda, S.** (2010). Trehalose extends longevity in the nematode *Caenorhabditis elegans*. *Aging cell,* **9**(4), 558-569.

**Houštěk, J., Pícková, A., Vojtíšková, A., Mráček, T., Pecina, P., and Ješina, P.** (2006). Mitochondrial diseases and genetic defects of ATP synthase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* **1757**(9), 1400-1405.

**Houthoofd, K., Braeckman, B. P., Johnson, T. E., and Vanfleteren, J. R.** (2003). Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in *Caenorhabditis elegans*. *Exp. Gerontol.,* **38**(9), 947-954.

**Houthoofd, K., Braeckman, B. P., Lenaerts, I., Brys, K., De Vreese, A., Van Eygen, S., and Vanfleteren, J. R.** (2002). Axenic growth up-regulates mass-specific metabolic rate, stress resistance, and extends life span in *Caenorhabditis elegans*. *Exp. Gerontol.,* **37**(12), 1371-1378.

**Houthoofd, K., Braeckman, B. P., Lenaerts, I., Brys, K., Matthijssens, F., De Vreese, A., Van Eygen, S., and Vanfleteren, J. R.** (2005). DAF-2 pathway mutations and food restriction in aging *Caenorhabditis elegans* differentially affect metabolism. *Neurobiol. Aging,* **5**(26), 689-696.

**Houtkooper, R. H., Mouchiroud, L., Ryu, D., Moullan, N., Katsyuba, E., Knott, G., Williams, R. W., and Auwerx, J.** (2013). Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature,* **497**(7450), 451-457.

**Hsiao, J.-Y., Chen, C.-Y., Yang, M.-J., and Ho, H.-C.** (2013). Live and dead GFP-tagged bacteria showed indistinguishable fluorescence in *Caenorhabditis elegans* gut. *Journal of Microbiology,* **51**(3), 367-372.

**Hsu, A.-L., Murphy, C. T., and Kenyon, C.** (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science,* **300**(5622), 1142-1145.

**Huang, C., Xiong, C., and Kornfeld, K.** (2004). Measurements of age-related changes of physiological processes that predict lifespan of *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.,* **101**(21), 8084-8089.

**Huang, J.-M., La Ragione, R. M., Nunez, A., and Cutting, S. M.** (2008). Immunostimulatory activity of *Bacillus* spores. *FEMS Immunol. Med. Microbiol.,* **53**(2), 195-203.

**Hughes, S. E., Evason, K., Xiong, C., and Kornfeld, K.** (2007). Genetic and Pharmacological Factors That Influence Reproductive Aging in Nematodes. *PLOS Genetics,* **3**(2), e25.

**Humbert, R., Brusilow, W., Gunsalus, R., Klionsky, D., and Simoni, R.** (1983). *Escherichia coli* mutants defective in the *uncH* gene. *J. Bacteriol.,* **153**(1), 416-422.

**Iatsenko, I., Yim, J. J., Schroeder, F. C., and Sommer, R. J.** (2014). *B. subtilis* GS67 Protects *C. elegans* from Gram-Positive Pathogens via Fengycin-Mediated Microbial Antagonism. *Curr. Biol.,* **24**(22), 2720-2727.

**Ingram, D. K., and Roth, G. S.** (2015). Calorie restriction mimetics: can you have your cake and eat it, too? *Ageing Res. Rev.,* **20**, 46-62.

**Iwamoto, A., Omote, H., Hanada, H., Tomioka, N., Itai, A., Maeda, M., and Futai, M.** (1991). Mutations in Ser174 and the glycine-rich sequence (Gly149, Gly150, and

Thr156) in the beta subunit of *Escherichia coli* H (+)-ATPase. *J. Biol. Chem.,* **266**(25), 16350-16355.

**Jansson, H.-B.** (1994). Adhesion of Conidia of *Drechmeria coniospora* to *Caenorhabditis elegans* Wild Type and Mutants. *J. Nematol.,* **26**(4), 430-435.

**Jensen, P. R., and Michelsen, O.** (1992). Carbon and energy metabolism of *atp* mutants of *Escherichia coli*. *J. Bacteriol.,* **174**(23), 7635-7641.

**Jiménez, J. L. B., and da Silva, A. P.** (2015). *Caenorhabditis elegans* longevity when fed with *Bacillus subtilis* mutants. *Biosaia: Revista de los másteres de Biotecnología Sanitaria y Biotecnología Ambiental, Industrial y Alimentaria,* **1**(4)

**Johnson, S. C., Rabinovitch, P. S., and Kaeberlein, M.** (2013). mTOR is a key modulator of ageing and age-related disease. *Nature,* **493**(7432), 338-345.

**Johnson, T. E., Mitchell, D. H., Kline, S., Kemal, R., and Foy, J.** (1984). Arresting development arrests aging in the nematode *Caenorhabditis elegans*. *Mech. Ageing Dev.,* **28**

**Kaeberlein, T. L., Smith, E. D., Tsuchiya, M., Welton, K. L., Thomas, J. H., Fields, S., Kennedy, B. K., and Kaeberlein, M.** (2006). Lifespan extension in *Caenorhabditis elegans* by complete removal of food. *Aging Cell,* **5**(6), 487-494.

**Kaletsky, R., and Murphy, C. T.** (2010). The role of insulin/IGF-like signaling in *C. elegans* longevity and aging. *Disease Models & Mechanisms,* **3**(7-8), 415-419.

**Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., and Ahringer, J.** (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biology,* **2**(1), 1-0002.

**Kapahi, P., Chen, D., Rogers, A. N., Katewa, S. D., Li, P. W.-L., Thomas, E. L., and Kockel, L.** (2010). With TOR, Less Is More: A Key Role for the Conserved Nutrient-Sensing TOR Pathway in Aging. *Cell Metab.,* **11**(6), 453-465.

**Kapahi, P., Kaeberlein, M., and Hansen, M.** (2017). Dietary restriction and lifespan: Lessons from invertebrate models. *Ageing Res. Rev.,* **39**, 3-14.

**Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., and Duran, C.** (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* **28**(12), 1647-1649.

**Kenyon, C., Chang, J., Gensch, E., and Rudner, A.** (1993). A *C. elegans* mutant that lives twice as long as wild type. *Nature,* **366**(6454), 461-464.

**Kim, D. H.** (2013). Bacteria and the aging and longevity of *Caenorhabditis elegans*. *Annu. Rev. Genet.,* **47**, 233-246.

**Kim, Y., and Mylonakis, E.** (2012). *Caenorhabditis elegans* immune conditioning with the probiotic bacterium *Lactobacillus acidophilus* strain NCFM enhances gram-positive immune responses. *Infect. Immun.,* **80**(7), 2500-2508.

**Kimura, K., Tanaka, N., Nakamura, N., Takano, S., and Ohkuma, S.** (2007). Knockdown of Mitochondrial Heat Shock Protein 70 Promotes Progeria-like Phenotypes in *Caenorhabditis elegans*. *J. Biol. Chem.,* **282**(8), 5910-5918.

**Kirkwood, T. B.** (2005). Understanding the odd science of aging. *Cell,* **120**(4), 437-447.

**Kirkwood, T. B. L.** (2002). Evolution of ageing. *Mech. Ageing Dev.,* **123**(7), 737-745.

**Kishimoto, S., Uno, M., Okabe, E., Nono, M., and Nishida, E.** (2017). Environmental stresses induce transgenerationally inheritable survival advantages via germline-to-soma communication in *Caenorhabditis elegans*. *Nature Communications,* **8**, 14031.

**Klass, M. R.** (1977). Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. *Mech. Ageing Dev.,* **6**, 413-429.

**Klionsky, D. J., Brusilow, W. S., and Simoni, R. D.** (1984). In vivo evidence for the role of the epsilon subunit as an inhibitor of the proton-translocating ATPase of *Escherichia coli*. *J. Bacteriol.,* **160**(3), 1055-1060.

**Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., and Bessieres, P.** (2003). Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci. U.S.A.,* **100**(8), 4678-4683.

**Koch-Koerfges, A., Kabus, A., Ochrombel, I., Marin, K., and Bott, M.** (2012). Physiology and global gene expression of a *Corynebacterium glutamicum* ΔF1FO-ATP synthase mutant devoid of oxidative phosphorylation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* **1817**(2), 370-380.

**Kolodkin-Gal, I., Elsholz, A. K. W., Muth, C., Girguis, P. R., Kolter, R., and Losick, R.** (2013). Respiration control of multicellularity in *Bacillus subtilis* by a complex of the cytochrome chain with a membrane-embedded histidine kinase. *Genes Dev.,* **27**(8), 887-899.

**Kunst, F., Ogasawara, N., Moszer, I., and Albertini, A.** (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature,* **390**(6657), 249.

**La Ragione, R. M., and Woodward, M. J.** (2003). Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype *Enteritidis* and *Clostridium perfringens* in young chickens. *Vet. Microbiol.,* **94**(3), 245-256.

**Laaberki, M.-H., and Dworkin, J.** (2008). Role of Spore Coat Proteins in the Resistance of *Bacillus subtilis* Spores to *Caenorhabditis elegans* Predation. *J. Bacteriol.,* **190**(18), 6197-6203.

**Labbadia, J., Brielmann, R. M., Neto, M. F., Lin, Y. F., Haynes, C. M., and Morimoto, R. I.** (2017). Mitochondrial stress restores the heat shock response and prevents proteostasis collapse during aging. *Cell reports,* **21**(6), 1481-1494.

**Lakowski, B., and Hekimi, S.** (1998). The genetics of caloric restriction in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.,* **95**(22), 13091-13096.

**Lee, C., and Longo, V.** (2016). Dietary restriction with and without caloric restriction for healthy aging. *F1000Research,* **5**(F1000 Faculty Rev), 117.

**Lee, D., Jeong, D.-E., Son, H. G., Yamaoka, Y., Kim, H., Seo, K., Khan, A. A., Roh, T.-Y., Moon, D. W., and Lee, Y.** (2015). SREBP and MDT-15 protect *C. elegans* from glucose-induced accelerated aging by preventing accumulation of saturated fat. *Genes Dev.,* **29**(23), 2490-2503.

**Lee, G. D., Wilson, M. A., Zhu, M., Wolkow, C. A., de Cabo, R., Ingram, D. K., and Zou, S.** (2006). Dietary deprivation extends lifespan in *Caenorhabditis elegans*. *Aging cell,* **5**(6), 515-524.

**Lee, J., Kwon, G., and Lim, Y.-H.** (2015). Elucidating the Mechanism of Weissella-dependent Lifespan Extension in *Caenorhabditis elegans*. *Scientific Reports,* **5**, 17128.

**Lee, K., and Mylonakis, E.** (2017). An Intestine-Derived Neuropeptide Controls Avoidance Behavior in *Caenorhabditis elegans*. *Cell Rep,* **20**(10), 2501-2512.

**Lee, R. C., Feinbaum, R. L., and Ambros, V.** (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell,* **75**(5), 843-854.

**Lee, S.-J., Hwang, A. B., and Kenyon, C.** (2010). Inhibition of Respiration Extends *C. elegans* Life Span via Reactive Oxygen Species that Increase HIF-1 Activity. *Curr. Biol.,* **20**(23), 2131-2136.

**Lee, S.-J., Murphy, C. T., and Kenyon, C.** (2009). Glucose Shortens the Life Span of *C. elegans* by Downregulating DAF-16/FOXO Activity and Aquaporin Gene Expression. *Cell Metab.,* **10**(5), 379-391.

**Lee, W.-J., and Hase, K.** (2014). Gut microbiota-generated metabolites in animal health and disease. *Nat. Chem. Biol.,* **10**(6), 416-424.

**Lefevre, M., Racedo, S. M., Ripert, G., Housez, B., Cazaubiel, M., Maudet, C., Jüsten, P., Marteau, P., and Urdaci, M. C.** (2015). Probiotic strain *Bacillus subtilis* CU1 stimulates immune system of elderly during common infectious disease period: a randomized, double-blind placebo-controlled study. *Immunity & Ageing : I & A,* **12**, 24.

**Leiers, B., Kampkötter, A., Grevelding, C. G., Link, C. D., Johnson, T. E., and Henkle-Dührsen, K.** (2003). A stress-responsive glutathione S-transferase confers resistance to oxidative stress in *Caenorhabditis elegans*. *Free Radical Biology and Medicine,* **34**(11), 1405-1415.

**Leiser, S. F., Jafari, G., Primitivo, M., Sutphin, G. L., Dong, J., Leonard, A., Fletcher, M., and Kaeberlein, M.** (2016). Age-associated vulval integrity is an important marker of nematode healthspan. *Age,* **38**(5-6), 419-431.

**Lenaerts, I., Walker, G. A., Van Hoorebeke, L., Gems, D., and Vanfleteren, J. R.** (2008). Dietary restriction of *Caenorhabditis elegans* by axenic culture reflects nutritional requirement for constituents provided by metabolically active microbes. *J Gerontol A Biol Sci Med Sci,* **63**(3), 242-252.

**Lezzerini, M., van de Ven, K., Veerman, M., Brul, S., and Budovskaya, Y. V.** (2015). Specific RNA Interference in *Caenorhabditis elegans* by Ingested dsRNA Expressed in *Bacillus subtilis*. **10**(4), e0124508.

**Li, H., Liu, X., Wang, D., Su, L., Zhao, T., Li, Z., Lin, C., Zhang, Y., Huang, B., Lu, J., and Li, X.** (2017). O-GlcNAcylation of SKN-1 modulates the lifespan and oxidative stress resistance in *Caenorhabditis elegans*. *Sci Rep,* **7**, 43601.

**Libert, S., Zwiener, J., Chu, X., Vanvoorhies, W., Roman, G., and Pletcher, S. D.** (2007). Regulation of *Drosophila* life span by olfaction and food-derived odors. *Science,* **315**(5815), 1133-1137.

**Liggett, M. R., Hoy, M. J., Mastroianni, M., and Mondoux, M. A.** (2015). High-glucose diets have sex-specific effects on aging in *C. elegans*: toxic to hermaphrodites but beneficial to males. *Aging (Albany NY),* **7**(6), 383-387.

**Lin, K., Hsin, H., Libina, N., and Kenyon, C.** (2001). Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat. Genet.,* **28**(2), 139.

**Link, C. D., and Johnson, C. J.** (2002). Reporter transgenes for study of oxidant stress in *Caenorhabditis elegans*. In *Methods Enzymol.,* Vol. 353, pp. 497-505. Edited by C. K. Sen and L. Packer. Elsevier.

**Liu, X., Jiang, N., Hughes, B., Bigras, E., Shoubridge, E., and Hekimi, S.** (2005). Evolutionary conservation of the *clk-1*-dependent mechanism of longevity: loss of *mclk1* increases cellular fitness and lifespan in mice. *Genes Dev.,* **19**(20), 2424-2434.

**Lucanic, M., Garrett, T., Yu, I., Calahorro, F., Asadi Shahmirzadi, A., Miller, A., Gill, M. S., Hughes, R. E., Holden-Dye, L., and Lithgow, G. J.** (2016). Chemical activation of a food deprivation signal extends lifespan. *Aging Cell,* **15**(5), 832-841.

**MacNeil, L. T., Watson, E., Arda, H. E., Zhu, L. J., and Walhout, A. J.** (2013). Diet-induced developmental acceleration independent of TOR and insulin in *C. elegans*. *Cell,* **153**(1), 240-252.

**Maglioni, S., Arsalan, N., Franchi, L., Hurd, A., Opipari, A. W., Glick, G. D., and Ventura, N.** (2015). An automated phenotype-based microscopy screen to identify pro-longevity interventions acting through mitochondria in *C. elegans*. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* **1847**(11), 1469-1478.

**Maier, W., Adilov, B., Regenass, M., and Alcedo, J.** (2010). A Neuromedin U Receptor Acts with the Sensory System to Modulate Food Type-Dependent Effects on *C. elegans* Lifespan. *PLoS Biol.,* **8**(5), e1000376.

**Mair, W., Panowski, S. H., Shaw, R. J., and Dillin, A.** (2009). Optimizing Dietary Restriction for Genetic Epistasis Analysis and Gene Discovery in *C. elegans*. *PLOS ONE,* **4**(2), e4535.

**Majumdar, R., Rajasekaran, K., and Cary, J. W.** (2017). RNA interference (RNAi) as a potential tool for control of mycotoxin contamination in crop plants: concepts and considerations. *Frontiers in plant science,* **8**, 200.

**Maman, M., Marques, F. C., Volovik, Y., Dubnikov, T., Bejerano-Sagie, M., and Cohen, E.** (2013). A neuronal GPCR is critical for the induction of the heat shock response in the nematode *C. elegans*. *J. Neurosci.,* **33**(14), 6102-6111.

**McKenney, P. T., Driks, A., and Eichenberger, P.** (2013). The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat. Rev. Microbiol.,* **11**(1), 33.

**Medawar, P. B.** (1952). An unsolved problem of biology. London, UK: H. K. Lewis.

**Mielich-Süss, B., and Lopez, D.** (2015). Molecular mechanisms involved in *Bacillus subtilis* biofilm formation. *Environmental microbiology,* **17**(3), 555-565.

**Miller, D. L., and Roth, M. B.** (2007). Hydrogen sulfide increases thermotolerance and lifespan in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.,* **104**(51), 20618-20622.

**Mobbs, C. V., Mastaitis, J., Zhang, M., Isoda, F., Cheng, H., and Yen, K.** (2007). Glucose Hysteresis as a Mechanism in Dietary Restriction, Aging and Disease. *Interdiscip. Top. Gerontol.,* **35**, 39-68.

**Mondoux, M. A., Love, D. C., Ghosh, S. K., Fukushige, T., Bond, M., Weerasinghe, G. R., Hanover, J. A., and Krause, M. W.** (2011). O-Linked-N-Acetylglucosamine Cycling and Insulin Signaling Are Required for the Glucose Stress Response in *Caenorhabditis elegans*. *Genetics,* **188**(2), 369-382.

**Montalvo-Katz, S., Huang, H., Appel, M. D., Berg, M., and Shapira, M.** (2013). Association with Soil Bacteria Enhances p38-Dependent Infection Resistance in *Caenorhabditis elegans*. *Infect. Immun.,* **81**(2), 514.

**Moore, B. T., Jordan, J. M., and Baugh, L. R.** (2013). WormSizer: High-throughput Analysis of Nematode Size and Shape. *PLOS ONE,* **8**(2), e57142.

**Most, J., Tosti, V., Redman, L. M., and Fontana, L.** (2017). Calorie restriction in humans: An update. *Ageing Res. Rev.,* **39**, 36-45.

**Mouchiroud, L., Molin, L., Kasturi, P., Triba, M. N., Dumas, M. E., Wilson, M. C., Halestrap, A. P., Roussel, D., Masse, I., Dallière, N., Ségalat, L., Billaud, M., and Solari, F.** (2011). Pyruvate imbalance mediates metabolic reprogramming and mimics lifespan extension by dietary restriction in *Caenorhabditis elegans*. *Aging Cell,* **10**(1), 39-54.

**Mracek, T., Pecina, P., Vojtiskova, A., Kalous, M., Sebesta, O., and Houstek, J.** (2006). Two components in pathogenic mechanism of mitochondrial ATPase deficiency: energy deprivation and ROS production. *Exp. Gerontol.,* **41**(7), 683-687.

**Murakami, H., Bessinger, K., Hellmann, J., and Murakami, S.** (2005). Aging-dependent and -independent modulation of associative learning behavior by insulin/insulin-like growth factor-1 signal in *Caenorhabditis elegans*. *J. Neurosci.,* **25**(47), 10894-10904.

**Nakagawa, H., Shiozaki, T., Kobatake, E., Hosoya, T., Moriya, T., Sakai, F., Taru, H., and Miyazaki, T.** (2016). Effects and mechanisms of prolongevity induced by *Lactobacillus gasseri* SBT2055 in *Caenorhabditis elegans*. *Aging Cell,* **15**(2), 227-236.

**Nehrke, K.** (2003). A reduction in intestinal cell pHi due to loss of the *Caenorhabditis elegans* Na+/H+ exchanger NHX-2 increases life span. *J. Biol. Chem.,* **278**(45), 44657-44666.

**Neilson, A.** (2017). *The Effects of Respiratory-deficient Bacterial Diets on Lifespan and Stress Resistance in* Caenorhabditis elegans. (Unpublished Master's (MBio) thesis). University of Warwick, Coventry.

**Newell Stamper, B. L., Cypser, J. R., Kechris, K., Kitzenberg, D. A., Tedesco, P. M., and Johnson, T. E.** (2018). Movement decline across lifespan of *Caenorhabditis elegans* mutants in the insulin/insulin-like signaling pathway. *Aging cell,* **17**(1), e12704.

**Niedzwiecka, K., Tisi, R., Penna, S., Lichocka, M., Plochocka, D., and Kucharczyk, R.** (2018). Two mutations in mitochondrial ATP6 gene of ATP synthase, related to human cancer, affect ROS, calcium homeostasis and mitochondrial permeability transition in yeast. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* **1865**(1), 117-131.

**Noda, S., Takezawa, Y., Mizutani, T., Asakura, T., Nishiumi, E., Onoe, K., Wada, M., Tomita, F., Matsushita, K., and Yokota, A.** (2006). Alterations of cellular physiology in *Escherichia coli* in response to oxidative phosphorylation impaired by defective F1-ATPase. *J. Bacteriol.,* **188**(19), 6869-6876.

**Onken, B., and Driscoll, M.** (2010). Metformin Induces a Dietary Restriction-Like State and the Oxidative Stress Response to Extend *C. elegans* Healthspan via AMPK, LKB1, and SKN-1. *PLoS ONE,* **5**(1), e8758.

**Palmer, M. R., and Sackton, T. B.** (2003). The effects of dietary coenzyme Q on *Drosophila* life span. *Aging Cell,* **2**(6), 335-339.

**Pang, S., and Curran, S. P.** (2014). Adaptive capacity to bacterial diet modulates aging in *C. elegans*. *Cell Metab.,* **19**(2), 221-231.

**Panowski, S. H., Wolff, S., Aguilaniu, H., Durieux, J., and Dillin, A.** (2007). PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans*. *Nature,* **447**(7144), 550.

**Park, H.-E. H., Jung, Y., and Lee, S.-J. V.** (2017). Survival assays using *Caenorhabditis elegans*. *Mol. Cells,* **40**(2), 90-99.

**Parsek, M. R., and Greenberg, E. P.** (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol.,* **13**(1), 27-33.

**Pawlikowska, L., Hu, D., Huntsman, S., Sung, A., Chu, C., Chen, J., Joyner, A. H., Schork, N. J., Hsueh, W. C., and Reiner, A. P.** (2009). Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. *Aging cell,* **8**(4), 460-472.

**Permpoonpattana, P., Hong, H. A., Khaneja, R., and Cutting, S. M.** (2012). Evaluation of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient. *Beneficial microbes,* **3**(2), 127-135.

**Podshivalova, K., Kerr, R. A., and Kenyon, C.** (2017). How a Mutation that Slows Aging Can Also Disproportionately Extend End-of-Life Decrepitude. *Cell Reports,* **19**(3), 441-450.

**Poormontaseri, M., Hosseinzadeh, S., Shekarforoush, S. S., and Kalantari, T.** (2017). The effects of probiotic *Bacillus subtilis* on the cytotoxicity of *Clostridium perfringens* type a in Caco-2 cell culture. *BMC Microbiol.,* **17**(1), 150.

**Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A., and Cerón, J.** (2012). Basic *Caenorhabditis elegans* Methods: Synchronization and Observation. *Journal of Visualized Experiments : JoVE,*(64), 4019.

**Portal-Celhay, C., and Blaser, M. J.** (2012). Competition and Resilience between Founder and Introduced Bacteria in the *Caenorhabditis elegans* Gut. *Infect. Immun.,* **80**(3), 1288-1299.

**Portal-Celhay, C., Bradley, E. R., and Blaser, M. J.** (2012). Control of intestinal bacterial proliferation in regulation of lifespan in *Caenorhabditis elegans*. *BMC Microbiol.,* **12**(1), 49.

**Preiss, L., Hicks, D. B., Suzuki, S., Meier, T., and Krulwich, T. A.** (2015). Alkaliphilic Bacteria with Impact on Industrial Applications, Concepts of Early Life Forms, and Bioenergetics of ATP Synthesis. *Frontiers in Bioengineering and Biotechnology,* **3**(75)

**Ramos, H. C., Hoffmann, T., Marino, M., Nedjari, H., Presecan-Siedel, E., Dreesen, O., Glaser, P., and Jahn, D.** (2000). Fermentative metabolism of *Bacillus subtilis*: physiology and regulation of gene expression. *J. Bacteriol.,* **182**(11), 3072-3080.

**Raynes, R., Leckey, B. D., Nguyen, K., and Westerheide, S. D.** (2012). Heat shock and caloric restriction have a synergistic effect on the heat shock response in a *sir2. 1*-dependent manner in *Caenorhabditis elegans*. *J. Biol. Chem.,* **287**(34), 29045-29053.

**Rea, S. L., Ventura, N., and Johnson, T. E.** (2007). Relationship Between Mitochondrial Electron Transport Chain Dysfunction, Development, and Life Extension in *Caenorhabditis elegans*. *PLoS Biol.,* **5**(10), e259.

**Redman, L. M., and Ravussin, E.** (2011). Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. *Antioxid. Redox Signal.,* **14**(2), 275-287.

**Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R., and Ruvkun, G.** (2000). The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature,* **403**(6772), 901-906.

**Richards, J. L., Yap, Y. A., McLeod, K. H., Mackay, C. R., and Mariño, E.** (2016). Dietary metabolites and the gut microbiota: an alternative approach to control inflammatory and autoimmune diseases. *Clinical & translational immunology,* **5**(5), e82.

**Ristow, M., and Schmeisser, S.** (2011). Extending life span by increasing oxidative stress. *Free Radical Biology and Medicine,* **51**(2), 327-336.

**Rodriguez, M., Snoek, L. B., De Bono, M., and Kammenga, J. E.** (2013). Worms under stress: *C. elegans* stress response and its relevance to complex human disease and aging. *Trends Genet.,* **29**(6), 367-374.

**Rooney, J. P., Luz, A. L., González-Hunt, C. P., Bodhicharla, R., Ryde, I. T., Anbalagan, C., and Meyer, J. N.** (2014). Effects of 5'-Fluoro-2-deoxyuridine on Mitochondrial Biology in *Caenorhabditis elegans*. *Exp. Gerontol.,* **56**, 69-76.

**Runkel, E. D., Liu, S., Baumeister, R., and Schulze, E.** (2013). Surveillance-Activated Defenses Block the ROS-Induced Mitochondrial Unfolded Protein Response. *PLoS Genet,* **9**(3), e1003346.

**Saez-Lara, M. J., Gomez-Llorente, C., Plaza-Diaz, J., and Gil, A.** (2015). The Role of Probiotic Lactic Acid Bacteria and Bifidobacteria in the Prevention and Treatment of Inflammatory Bowel Disease and Other Related Diseases: A Systematic Review of Randomized Human Clinical Trials. *BioMed Research International,* **2015**, 505878.

**Said, G.** (2018). *The effects of a respiratory-deficient diet on modulation of ageing and stress-resistance in* Caenorhabditis elegans*.* (Unpublished Master's (MBio) thesis). University of Warwick, Coventry.

**Saiki, R., Lunceford, A. L., Bixler, T., Dang, P., Lee, W., Furukawa, S., Larsen, P. L., and Clarke, C. F.** (2008). Altered bacterial metabolism, not coenzyme Q content, is responsible for the lifespan extension in *Caenorhabditis elegans* fed an *Escherichia coli* diet lacking coenzyme Q. *Aging cell,* **7**(3), 291-304.

**Salim, C., and Rajini, P. S.** (2014). Glucose feeding during development aggravates the toxicity of the organophosphorus insecticide Monocrotophos in the nematode, *Caenorhabditis elegans*. *Physiol. Behav.,* **131**, 142-148.

**Salim, C., and Rajini, P. S.** (2017). Glucose-rich diet aggravates monocrotophos-induced dopaminergic neuronal dysfunction in *Caenorhabditis elegans*. *J. Appl. Toxicol.,* **37**(6), 772-780.

**Salinas, A. E., and Wong, M. G.** (1999). Glutathione S-transferases-a review. *Curr. Med. Chem.,* **6**(4), 279-310.

**Samuel, B. S., Rowedder, H., Braendle, C., Félix, M.-A., and Ruvkun, G.** (2016). *Caenorhabditis elegans* responses to bacteria from its natural habitats. *Proc. Natl. Acad. Sci. U. S. A.,* **113**(27), E3941-E3949.

**Sánchez-Blanco, A., and Kim, S. K.** (2011). Variable Pathogenicity Determines Individual Lifespan in *Caenorhabditis elegans*. *PLoS Genetics,* **7**(4), e1002047.

**Sánchez-Blanco, A., Rodríguez-Matellán, A., González-Paramás, A., González-Manzano, S., Kim, S. K., and Mollinedo, F.** (2016). Dietary and microbiome factors determine longevity in *Caenorhabditis elegans*. *Aging (Albany NY),* **8**(7), 1513-1530.

**Santana, M., Ionescu, M. S., Vertes, A., Longin, R., Kunst, F., Danchin, A., and Glaser, P.** (1994). *Bacillus subtilis* F0F1 ATPase: DNA sequence of the atp operon and characterization of *atp* mutants. *J. Bacteriol.,* **176**(22), 6802-6811.

**Saraswati, S., and Sitaraman, R.** (2015). Aging and the human gut microbiota—from correlation to causality. *Frontiers in microbiology,* **5**, 764.

**Sasahara, K. C., Heinzinger, N. K., and Barrett, E. L.** (1997). Hydrogen sulfide production and fermentative gas production by *Salmonella typhimurium* require F0F1 ATP synthase activity. *J. Bacteriol.,* **179**(21), 6736-6740.

**Scerbak, C., Vayndorf, E. M., Parker, J. A., Neri, C., Driscoll, M., and Taylor, B. E.** (2014). Insulin signaling in the aging of healthy and proteotoxically stressed mechanosensory neurons. *Frontiers in genetics,* **5**, 212.

**Schlotterer, A., Kukudov, G., Bozorgmehr, F., Hutter, H., Du, X., Oikonomou, D., Ibrahim, Y., Pfisterer, F., Rabbani, N., and Thornalley, P.** (2009). *C. elegans* as model for the study of high glucose–mediated life span reduction. *Diabetes,* **58**(11), 2450-2456.

**Schmeisser, S., Schmeisser, K., Weimer, S., Groth, M., Priebe, S., Fazius, E., Kuhlow, D., Pick, D., Einax, J. W., Guthke, R., Platzer, M., Zarse, K., and Ristow, M.** (2013). Mitochondrial hormesis links low-dose arsenite exposure to lifespan extension. *Aging Cell,* **12**(3), 508-517.

**Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M., and Ristow, M.** (2007). Glucose restriction extends *Caenorhabditis elegan*s life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab.,* **6**(4), 280-293.

**Selman, C.** (2014). Dietary restriction and the pursuit of effective mimetics. *Proc. Nutr. Soc.,* **73**(2), 260-270.

**Senchuk, M. M., Dues, D. J., and Van Raamsdonk, J. M.** (2017). Measuring Oxidative Stress in *Caenorhabditis elegans*: Paraquat and Juglone Sensitivity Assays. *Bio-protocol,* **7**(1), e2086.

**Seo, Y., Kingsley, S., Walker, G., Mondoux, M. A., and Tissenbaum, H. A.** (2018). Metabolic shift from glycogen to trehalose promotes lifespan and healthspan in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.,* **115**(12), E2791-E2800.

**Shapira, M.** (2017). Host–microbiota interactions in *Caenorhabditis elegans* and their significance. *Curr. Opin. Microbiol.,* **38**, 142-147.

**Shreiner, A. B., Kao, J. Y., and Young, V. B.** (2015). The gut microbiome in health and in disease. *Current opinion in gastroenterology,* **31**(1), 69-75.

**Shtonda, B. B., and Avery, L.** (2006). Dietary choice behavior in *Caenorhabditis elegans*. *The Journal of experimental biology,* **209**(Pt 1), 89-102.

**Smith, E. D., Kaeberlein, T. L., Lydum, B. T., Sager, J., Welton, K. L., Kennedy, B. K., and Kaeberlein, M.** (2008). Age- and calorie-independent life span extension from dietary restriction by bacterial deprivation in *Caenorhabditis elegans*. *BMC Dev. Biol.,* **8**(1), 1-13.

**Smolentseva, O., Gusarov, I., Gautier, L., Shamovsky, I., DeFrancesco, A. S., Losick, R., and Nudler, E.** (2017). Mechanism of biofilm-mediated stress resistance and lifespan extension in *C. elegans*. *Scientific Reports,* **7**(1), 7137.

**So, S., Miyahara, K., and Ohshima, Y.** (2011). Control of body size in *C. elegans* dependent on food and insulin/IGF-1 signal. *Genes Cells,* **16**(6), 639-651.

**Sonowal, R., Swimm, A., Sahoo, A., Luo, L., Matsunaga, Y., Wu, Z., Bhingarde, J. A., Ejzak, E. A., Ranawade, A., and Qadota, H.** (2017). Indoles from commensal bacteria extend healthspan. *Proc. Natl. Acad. Sci. U.S.A.,* **114**(36), E7506-E7515.

**Stein, L., Sternberg, P., Durbin, R., Thierry-Mieg, J., and Spieth, J.** (2001). WormBase: network access to the genome and biology of *Caenorhabditis elegans*. *Nucleic Acids Res.,* **29**(1), 82-86.

**Steinkraus, K. A., Smith, E. D., Davis, C., Carr, D., Pendergrass, W. R., Sutphin, G. L., Kennedy, B. K., and Kaeberlein, M.** (2008). Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in *Caenorhabditis elegans*. *Aging cell,* **7**(3), 394-404.

**Sterken, M. G., Snoek, L. B., Kammenga, J. E., and Andersen, E. C.** (2015). The laboratory domestication of *Caenorhabditis elegans*. *Trends in genetics : TIG,* **31**(5), 224-231.

**Sterlini, J. M., and Mandelstam, J.** (1969). Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. *Biochem. J.,* **113**(1), 29-37.

**Sun, G., Sharkova, E., Chesnut, R., Birkey, S., Duggan, M. F., Sorokin, A., Pujic, P., Ehrlich, S. D., and Hulett, F. M.** (1996). Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. *J. Bacteriol.,* **178**(5), 1374-1385.

**Svensk, E., Devkota, R., Ståhlman, M., Ranji, P., Rauthan, M., Magnusson, F., Hammarsten, S., Johansson, M., Borén, J., and Pilon, M.** (2016). *Caenorhabditis elegans* PAQR-2 and IGLR-2 Protect against Glucose Toxicity by Modulating Membrane Lipid Composition. *PLoS Genet,* **12**(4), e1005982.

**Tabara, H., Grishok, A., and Mello, C. C.** (1998). RNAi in *C. elegans*: soaking in the genome sequence. *Science,* **282**(5388), 430-431.

**Tain, L. S., Lozano, E., Sáez, A. G., and Leroi, A. M.** (2008). Dietary regulation of hypodermal polyploidization in *C. elegans*. *BMC Dev. Biol.,* **8**(1), 28.

**Tan, I. S., and Ramamurthi, K. S.** (2014). Spore formation in *Bacillus subtilis*. *Environmental microbiology reports,* **6**(3), 212-225.

**Tanaka, K., Henry, C. S., Zinner, J. F., Jolivet, E., Cohoon, M. P., Xia, F., Bidnenko, V., Ehrlich, S. D., Stevens, R. L., and Noirot, P.** (2013). Building the repertoire of dispensable chromosome regions in *Bacillus subtilis* entails major refinement of cognate large-scale metabolic model. *Nucleic Acids Res.,* **41**(1), 687-699.

**Tao, J., Wu, Q.-Y., Ma, Y.-C., Chen, Y.-L., and Zou, C.-G.** (2017). Antioxidant response is a protective mechanism against nutrient deprivation in *C. elegans*. *Scientific Reports,* **7**, 43547.

**Tauffenberger, A., Vaccaro, A., Aulas, A., Velde, C. V., and Parker, J. A.** (2012). Glucose delays age-dependent proteotoxicity. *Aging Cell,* **11**(5), 856-866.

**Tauffenberger, A., Vaccaro, A., and Parker, J. A.** (2016). Fragile lifespan expansion by dietary mitohormesis in *C. elegans*. *Aging (Albany NY),*

**Tawe, W. N., Eschbach, M.-L., Walter, R. D., and Henkle-Dührsen, K.** (1998). Identification of stress-responsive genes in *Caenorhabditis elegans* using RT-PCR differential display. *Nucleic Acids Res.,* **26**(7), 1621-1627.

**Thondamal, M., Witting, M., Schmitt-Kopplin, P., and Aguilaniu, H.** (2014). Steroid hormone signalling links reproduction to lifespan in dietary-restricted *Caenorhabditis elegans*. *Nature Communications,* **5**, 4879.

**Timmons, L., Court, D. L., and Fire, A.** (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene,* **263**(1), 103-112.

**Timmons, L., and Fire, A.** (1998). Specific interference by ingested dsRNA. *Nature,* **395**(6705), 854.

**Tissenbaum, H. A.** (2012). Genetics, life span, health span, and the aging process in *Caenorhabditis elegans*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* **67**(5), 503-510.

**Tissenbaum, H. A.** (2015). Using *C. elegans* for aging research. *Invertebr. Reprod. Dev.,* **59**(sup1), 59-63.

**Torgovnick, A., Schiavi, A., Maglioni, S., and Ventura, N.** (2013). Healthy aging: what can we learn from *Caenorhabditis elegans*? *Zeitschrift für Gerontologie und Geriatrie,* **46**(7), 623-628.

**Tsang, W. Y., Sayles, L. C., Grad, L. I., Pilgrim, D. B., and Lemire, B. D.** (2001). Mitochondrial respiratory chain deficiency in *Caenorhabditis elegans* results in developmental arrest and increased life span. *J. Biol. Chem.,* **276**(34), 32240-32246.

**Tullet, J., Green, J. W., Au, C., Benedetto, A., Thompson, M. A., Clark, E., Gilliat, A. F., Young, A., Schmeisser, K., and Gems, D.** (2017). The SKN-1/Nrf2 transcription factor can protect against oxidative stress and increase lifespan in *C. elegans* by distinct mechanisms. *Aging cell,* **16**(5), 1191-1194.

**Tullet, J. M. A., Araiz, C., Sanders, M. J., Au, C., Benedetto, A., Papatheodorou, I., Clark, E., Schmeisser, K., Jones, D., Schuster, E. F., Thornton, J. M., and Gems, D.** (2014). DAF-16/FoxO Directly Regulates an Atypical AMP-Activated Protein Kinase Gamma Isoform to Mediate the Effects of Insulin/IGF-1 Signaling on Aging in *Caenorhabditis elegans*. *PLOS Genetics,* **10**(2), e1004109.

**van Gestel, J., Vlamakis, H., and Kolter, R.** (2015). Division of Labor in Biofilms: the Ecology of Cell Differentiation. *Microbiology spectrum,* **3**(2), Mb-0002-2014.

**Van Gilst, M. R., Hadjivassiliou, H., and Yamamoto, K. R.** (2005). A *Caenorhabditis elegans* nutrient response system partially dependent on nuclear receptor NHR-49. *Proc. Natl. Acad. Sci. U. S. A.,* **102**(38), 13496-13501.

**Van Raamsdonk, J. M., and Hekimi, S.** (2011). FUdR causes a twofold increase in the lifespan of the mitochondrial mutant *gas-1*. *Mech. Ageing Dev.,* **132**(10), 519-521.

**Van Raamsdonk, J. M., and Hekimi, S.** (2012). Superoxide dismutase is dispensable for normal animal lifespan. *Proc. Natl. Acad. Sci. U.S.A.,* **109**(15), 5785-5790.

**van Tongeren, S. P., Slaets, J. P., Harmsen, H., and Welling, G. W.** (2005). Fecal microbiota composition and frailty. *Appl. Environ. Microbiol.,* **71**(10), 6438-6442.

**Vavrová, Ľ., Muchová, K., and Barák, I.** (2010). Comparison of different *Bacillus subtilis* expression systems. *Res. Microbiol.,* **161**(9), 791-797.

**Virk, B., Correia, G., Dixon, D. P., Feyst, I., Jia, J., Oberleitner, N., Briggs, Z., Hodge, E., Edwards, R., and Ward, J.** (2012). Excessive folate synthesis limits lifespan in the *C. elegans*: *E. coli* aging model. *BMC biology,* **10**(1), 67.

**Virk, B., Jia, J., Maynard, C. A., Raimundo, A., Lefebvre, J., Richards, S. A., Chetina, N., Liang, Y., Helliwell, N., and Cipinska, M.** (2016). Folate Acts in *E. coli* to Accelerate *C. elegans* Aging Independently of Bacterial Biosynthesis. *Cell reports,* **14**(7), 1611-1620.

**Vlamakis, H., Chai, Y., Beauregard, P., Losick, R., and Kolter, R.** (2013). Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat. Rev. Microbiol.,* **11**(3), 157.

**Wang, W., and Bechhofer, D. H.** (1997). *Bacillus subtilis* RNase III gene: cloning, function of the gene in *Escherichia coli*, and construction of *Bacillus subtilis* strains with altered *rnc* loci. *J. Bacteriol.,* **179**(23), 7379-7385.

**Watson, E., MacNeil, L. T., Arda, H. E., Zhu, L. J., and Walhout, A. J.** (2013). Integration of metabolic and gene regulatory networks modulates the C. elegans dietary response. *Cell,* **153**(1), 253-266.

**Watson, E., MacNeil, L. T., Ritter, A. D., Yilmaz, L. S., Rosebrock, A. P., Caudy, A. A., and Walhout, A. J.** (2014). Interspecies systems biology uncovers metabolites affecting *C. elegans* gene expression and life history traits. *Cell,* **156**(4), 759-770.

**Westers, H., Dorenbos, R., van Dijl, J. M., Kabel, J., Flanagan, T., Devine, K. M., Jude, F., Séror, S. J., Beekman, A. C., Darmon, E., Eschevins, C., de Jong, A., Bron, S., Kuipers, O. P., Albertini, A. M., Antelmann, H., Hecker, M., Zamboni, N., Sauer, U., Bruand, C., Ehrlich, D. S., Alonso, J. C., Salas, M., and Quax, W. J.** (2003). Genome Engineering Reveals Large Dispensable Regions in *Bacillus subtilis*. *Mol. Biol. Evol.,* **20**(12), 2076-2090.

**Whiteside, S. A., Razvi, H., Dave, S., Reid, G., and Burton, J. P.** (2015). The microbiome of the urinary tract—a role beyond infection. *Nature Reviews Urology,* **12**(2), 81.

**Wilhelm, T., Byrne, J., Medina, R., Kolundžić, E., Geisinger, J., Hajduskova, M., Tursun, B., and Richly, H.** (2017). Neuronal inhibition of the autophagy nucleation complex extends life span in post-reproductive *C. elegans*. *Genes Dev.,* **31**(15), 1561-1572.

**Willcox, B. J., Donlon, T. A., He, Q., Chen, R., Grove, J. S., Yano, K., Masaki, K. H., Willcox, D. C., Rodriguez, B., and Curb, J. D.** (2008). FOXO3A genotype is strongly associated with human longevity. *Proc. Natl. Acad. Sci. U.S.A.,* **105**(37), 13987-13992.

**Williams, G. C.** (1957). Pleiotropy, Natural Selection, and the Evolution of Senescence. *Evolution,* **11**(4), 398-411.

**Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D., and Lewis, J. D.** (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science,* **334**(6052), 105-108.

**Xiao, R., Chun, L., Ronan, E. A., Friedman, D. I., Liu, J., and Xu, X. S.** (2015). RNAi Interrogation of Dietary Modulation of Development, Metabolism, Behavior, and Aging in *C. elegans*. *Cell reports,* **11**(7), 1123-1133.

**Yang, S., Du, G., Chen, J., and Kang, Z.** (2017). Characterization and application of endogenous phase-dependent promoters in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.,* **101**(10), 4151-4161.

**Yang, W., and Hekimi, S.** (2010). A mitochondrial superoxide signal triggers increased longevity in *Caenorhabditis elegans*. *PLoS Biol.,* **8**(12), e1000556.

**Yanos, M. E., Bennett, C. F., and Kaeberlein, M.** (2012). Genome-Wide RNAi Longevity Screens in *Caenorhabditis elegans*. *Curr. Genomics,* **13**(7), 508-518.

**Ye, X., Li, P., Yu, Q., and Yang, Q.** (2013). *Bacillus subtilis* inhibition of enterotoxic *Escherichia coli*-induced activation of MAPK signaling pathways in Caco-2 cells. *Ann. Microbiol.,* **63**(2), 577-581.

**Yilmaz, L. S., and Walhout, A. J.** (2014). Worms, bacteria, and micronutrients: an elegant model of our diet. *Trends Genet.,* **30**(11), 496-503.

**Yokoyama, K., Fukumoto, K., Murakami, T., Harada, S.-i., Hosono, R., Wadhwa, R., Mitsui, Y., and Ohkuma, S.** (2002). Extended longevity of *Caenorhabditis elegans* by knocking in extra copies of *hsp70F*, a homolog of *mot-2* (mortalin)/*mthsp70/Grp75*. *FEBS Lett.,* **516**(1-3), 53-57.

**Yoneda, T., Benedetti, C., Urano, F., Clark, S. G., Harding, H. P., and Ron, D.** (2004). Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J. Cell Sci.,* **117**(18), 4055-4066.

**Yu, L., Yan, X., Ye, C., Zhao, H., Chen, X., Hu, F., and Li, H.** (2015). Bacterial Respiration and Growth Rates Affect the Feeding Preferences, Brood Size and Lifespan of *Caenorhabditis elegans*. *PLoS ONE,* **10**(7), e0134401.

**Yuan, Y., Kadiyala, C. S., Ching, T.-T., Hakimi, P., Saha, S., Xu, H., Yuan, C., Mullangi, V., Wang, L., and Fivenson, E.** (2012). Enhanced energy metabolism contributes to the extended life span of calorie-restricted *Caenorhabditis elegans*. *J. Biol. Chem.,* **287**(37), 31414-31426.

**Zanni, E., Laudenzi, C., Schifano, E., Palleschi, C., Perozzi, G., Uccelletti, D., and Devirgiliis, C.** (2015). Impact of a Complex Food Microbiota on Energy Metabolism in the Model Organism *Caenorhabditis elegans*. *BioMed Research International,* **2015**, 1-12.

**Zeigler, D. R., Prágai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M., and Perkins, J. B.** (2008). The Origins of 168, W23, and Other *Bacillus subtilis* Legacy Strains. *J. Bacteriol.,* **190**(21), 6983-6995.

**Zhang, W. B., Sinha, D. B., Pittman, W. E., Hvatum, E., Stroustrup, N., and Pincus, Z.** (2016). Extended twilight among isogenic *C. elegans* causes a disproportionate scaling between lifespan and health. *Cell systems,* **3**(4), 333-345.

**Zhang, Y., Chen, D., Smith, M. A., Zhang, B., and Pan, X.** (2012). Selection of reliable reference genes in *Caenorhabditis elegans* for analysis of nanotoxicity. *PloS one,* **7**(3), e31849.

**Zhao, L., Zhao, Y., Liu, R., Zheng, X., Zhang, M., Guo, H., Zhang, H., and Ren, F.** (2017). The Transcription Factor DAF-16 is Essential for Increased Longevity in *C. elegans* Exposed to *Bifidobacterium longum* BB68. *Scientific Reports,* **7**(1), 7408.

**Zhao, Y., Gilliat, A. F., Ziehm, M., Turmaine, M., Wang, H., Ezcurra, M., Yang, C., Phillips, G., McBay, D., and Zhang, W. B.** (2017). Two forms of death in ageing *Caenorhabditis elegans*. *Nature communications,* **8**, 15458.

**Zhao, Y., Zhao, L., Zheng, X., Fu, T., Guo, H., and Ren, F.** (2013). *Lactobacillus salivarius* strain FDB89 induced longevity in *Caenorhabditis elegans* by dietary restriction. *Journal of Microbiology,* **51**(2), 183-188.

**Zheng, J., Gao, C., Wang, M., Tran, P., Mai, N., Finley, J. W., Heymsfield, S. B., Greenway, F. L., Li, Z., Heber, D., Burton, J. H., Johnson, W. D., and Laine, R. A.** (2017). Lower Doses of Fructose Extend Lifespan in *Caenorhabditis elegans*. *Journal of Dietary Supplements,* **14**(3), 264-277.

**Zhou, K. I., Pincus, Z., and Slack, F. J.** (2011). Longevity and stress in *Caenorhabditis elegans*. *Aging (Albany NY),* **3**(8), 733-753.

**Zhou, X., Zhang, N., Xia, L., Li, Q., Shao, J., Shen, Q., and Zhang, R.** (2018). ResDE two-component regulatory system mediates oxygen limitation-induced biofilm formation in *Bacillus amyloliquefaciens* SQR9. *Appl. Environ. Microbiol.,* **84**(8), 02744-02717.

**Zhu, G., Yin, F., Wang, L., Wei, W., Jiang, L., and Qin, J.** (2016). Modeling type 2 diabetes-like hyperglycemia in *C. elegans* on a microdevice. *Integrative Biology,* **8**(1), 30-38.

**Zhu, Y., Eiteman, M. A., Altman, R., and Altman, E.** (2008). High glycolytic flux improves pyruvate production by a metabolically engineered *Escherichia coli* strain. *Appl. Environ. Microbiol.,* **74**(21), 6649-6655.

**Zhuang, J. J., and Hunter, C. P.** (2012). RNA interference in *Caenorhabditis elegans*: uptake, mechanism, and regulation. *Parasitology,* **139**(5), 560-573.

**Zimniak, P.** (2012). What is the Proximal Cause of Aging? *Frontiers in Genetics,* **3**, 189.

## Appendices

### Appendix A

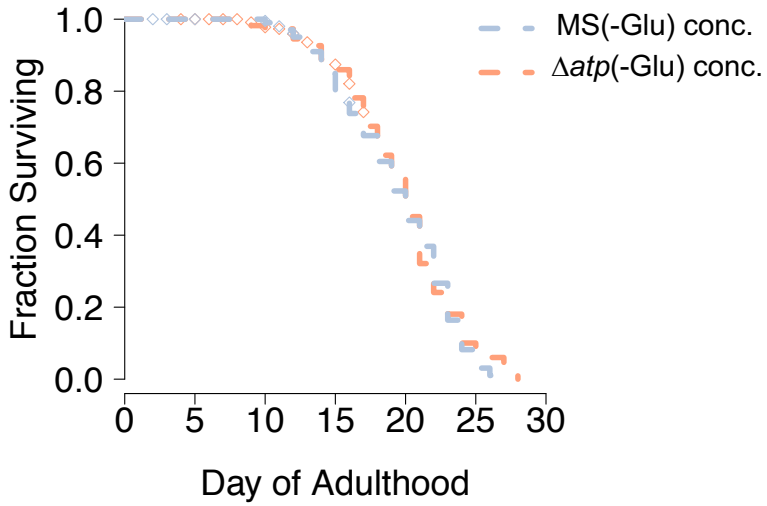



**Figure A1 Lifespan of *C. elegans* fed on *B. subtilis* MS(-Glu) or Δ*atp*(-Glu) (both concentrated, conc.).** There was no significant difference in the survival curves of animals fed on *B. s.* MS(-Glu) (N = 105) or *B. s.* Δ*atp*(-Glu) (N = 124) ($p$ = 0.42). Lifespan experiments were conducted at 20 °C. Diamond marks represent censoring. The log rank test was performed on Kaplan Meier survival curves.

**Table A1 Summary of lifespan assays in the absence of FUdR.** The figure panels to which the experiment refers are indicated. Shading indicates experiments conducted in parallel. The mean and median lifespan and standard error values were calculated from a log rank test from samples of at least 100 individuals each. (-Glu), minus glucose; (+Glu), plus glucose 2%. C, concentrated *B. subtilis* lawn. U, unconcentrated lawn. l, lost through escape; b, bagged; v, vulval explosion. Symbols indicate comparisons between groups. *p*, log-rank test on Kaplan-Meier survival curves with Holm-Sidak multiple comparison *p* -value correction. ns, no significant difference ($p > 0.05$).

| **Symbol** | **Figure** | **Strain** | ***B. subtilis* diet** | **C/U** | **Deaths/ total (% censored)** | **Censored** | **Mean±SEM (days)** | **Median (days)** | **Max (days)*** | ***p*-val / % increase ****** |
|---|---|---|---|---|---|---|---|---|---|---|
| ♦ | 3. 3A | N2 | MS L1(+Glu)*** | U | 78/100 (22) | 22 (11l; 6b; 5v) | 15.6 ± 0.5 | 15 | 23.4 | • < 0.0001 |
| • | 3. 3A | N2 | Δ*atp* L1(+Glu) | U | 87/102(15) | 15 (10l; 1b; 4v) | 23.7 ± 0.56 | 23 | 31.6 | ♦ < 0.0001 / +52<br>ο 0.633 (ns) |
| § | 3. 3B | N2 | MS(-Glu) | U | 99/121 (18) | 22 (1l; 2b; 19v) | 18.22 ± 0.42 | 18 | 25.92 | ◊ 0.2 (n.s.)<br>† 0.2 (n.s.)<br>ο < 0.0001 |
| ◊ | 3. 3B | N2 | MS(+Glu) | U | 124/131 (5) | 7 (1l; 4b; 2v) | 19.33 ± 0.36 | 20 | 26.15 | § 0.814 (n.s.)<br>† 0.2 (n.s.)<br>ο < 0.0001<br>♦ < 0.0001 / +24 |
| † | 3. 3B | N2 | Δ*atp*(-Glu) | C | 96/121 (21) | 25 (2l; 3b; 20v) | 19.46 ± 0.36 | 19 | 25.17 | § 0.2 (n.s.)<br>◊ 0.9 (n.s.)<br>ο 0.2 (n.s.) |

| Symbol | Figure | Strain | *B. subtilis* diet | C/U | Deaths/ total (% censored) | Censored | Mean±SEM (days) | Median (days) | Max (days)* | *p*-val / % increase ** |
|---|---|---|---|---|---|---|---|---|---|---|
| o | 3. 3B | N2 | Δ*atp*(+Glu) | C-U**** | 85/122 (30) | 37 (24l; 12b; 1v) | 23.60 ± 0.66 | 24 | 32.17 | § < 0.0001 /+30<br>◊ < 0.0001 /+22<br>† < 0.0001 /+21<br>• 0.633 (n.s.) |
| # | A1 | N2 | MS(-Glu) | C | 98/105 (7) | 7 (1l; 4b; 2v) | 19.37 ± 0.41 | 20 | 25.09 | ¶ 0.42 (n.s.) |
| ¶ | A1 | N2 | Δ*atp*(-Glu) | C | 101/124 (19) | 23 (2l; 5b; 16v) | 19.69 ± 0.41 | 20 | 26.33 | # 0.42 (n.s.) |
| ◊ ***** | 3. 4 | N2 | MS(+Glu) | C-U | 73/76 (4) | 3 | 19.307±0.679 | 21±0.309 | - | ♦ < 0.0001<br>o < 0.01 / +4<br>◊ < 0.0001 / +13 |
| ♦ ***** | 3. 4 | N2 | Δ*atp*(+Glu) | C-U | 82/101 (19) | 19 | 27.104±0.788 | 27±1.321 | - | ◊ < 0.0001 / +40<br>o < 0.0001 / +47<br>• < 0.0001 / +58 |
| o ***** | 3. 4 | N2 | MS(-Glu) | C-U | 69/76 (9) | 7 | 18.487±0.379 | 19±0.282 | - | ◊ < 0.01<br>♦ < 0.0001<br>• 0.178 (n.s.) |
| • ***** | 3. 4 | N2 | Δ*atp*(-Glu) | C-U | 86/105 (18) | 19 | 17.138±0.494 | 17±0.660 | - | ◊ < 0.001<br>♦ < 0.0001<br>o 0.178 |

* Maximum lifespan was calculated as the mean of the top 10% longest-lived individuals in each group.

** Where the diet extended lifespan, the percentage increase in mean lifespan (%) is given.

*** L1+Glu: Nematodes were exposed to glucose from L1 onwards, as opposed to L4 onwards.

**** C-U, nematodes were fed on concentrated lawns in the absence of glucose until the L4 stage, at which point they were transferred to unconcentrated lawns in the presence of glucose.

***** Data collected by another researcher (Neilson, 2017)

**Table A2 Summary of lifespan assays using *E. coli* DK8 (master strain) and DK8 Δ*unc* (lacking ATP synthase).** The figure panels to which the experiment refers are indicated. The mean and median lifespan and standard error values were calculated from a log rank test from samples of at least 111 individuals each. (-Glu), minus glucose; (+Glu), plus glucose 2%. C, concentrated *B. subtilis* lawn; U, unconcentrated lawn. Symbols indicate comparisons between groups. *p*, log-rank test on Kaplan-Meier survival curves with Holm-Sidak multiple comparison *p* -value correction. Where the diet extended lifespan in this comparison, the percentage increase in mean lifespan (%) is given. Data was collected by another researcher (Neilson, 2017).

| Symbol | Fig. | Strain | *E. coli* diet | C/U | Deaths/total (% censored) | Censored | Mean ± SE (days) | Median ± SE (days) | *p*-val / % increase * |
|---|---|---|---|---|---|---|---|---|---|
| ◊ | 3. 5 | N2 | DK8(+Glu) | C | 93/111 (16) | 18 | 13.009±0.381 | 15.000±0.446 | ♦ < 0.01<br>o < 0.0001<br>• < 0.0001 |
| ♦ | 3. 5 | N2 | DK8 Δ*unc*(+Glu) | C | 99/118 (16) | 19 | 14.733±0.304 | 17.000±0.382 | ◊ < 0.01 / +13<br>o < 0.0001<br>• < 0.0001 |
| o | 3. 5 | N2 | DK8(-Glu) | C | 102/120 (15) | 18 | 16.918±0.494 | 20.000±0.356 | ◊ < 0.0001 / +30<br>♦ < 0.0001 / +15<br>• < 0.0001 |
| • | 3. 5 | N2 | DK8 Δ*unc*(-Glu) | C | 104/120 (13) | 16 | 21.101±0.536 | 26.000±1.074 | ◊ < 0.0001 / +62<br>♦ < 0.0001 /+43<br>o < 0.0001 /+25 |

* Where the diet extended lifespan in this comparison, the percentage increase in mean lifespan (%) is given.

**Table A3 Summary of lifespan assays in the presence of FUdR.** The figure panels to which the experiment refers are indicated. Shading indicates experiments conducted in parallel. The mean and median lifespan and standard error values were calculated from a log rank test from samples of at least 100 individuals each. (-Glu), minus glucose; (+Glu), plus glucose 2%.C, concentrated *B. subtilis* lawn. U, unconcentrated lawn. l, lost through escape; b, bagged; v, vulval explosion. Symbols indicate comparisons between groups. *p*, log-rank test on Kaplan-Meier survival curves with Holm-Sidak multiple comparison *p* -value correction. n.s., no significant difference ($p > 0.05$).

| Symbol | Figure | Strain | *B. subtilis* diet | C/U | Deaths/ total (% censored) | Mean±SEM (days) | Median (days) | Max (days)* | *p*-val / % increase ** |
|---|---|---|---|---|---|---|---|---|---|
| ◊ | 3. 6A | N2 | MS (-Glu) | U | 102/122 (16) | 21.1 ± 0.7 | 17 | 35.5 | ♦ 0.0017<br>o 0.0154<br>• < 0.0001 |
| ♦ | 3. 6A | N2 | MS(L1+Glu)*** | U | 88/120 (27) | 24.6 ± 0.8 | 23 | 37.8 | ◊ 0.0017/+17<br>o 0.9163<br>• < 0.0001 |
| o | 3. 6A | N2 | Δ*atp*(-Glu) | U | 115/121 (5) | 25.7 ± 0.38 | 26 | 33 | ◊ 0.0154/+22<br>♦ 0.9163<br>• < 0.0001 |
| • | 3. 6A | N2 | Δ*atp*(L1+Glu) | U | 117/121 (3) | 31.6 ± 0.57 | 32 | 40.9 | ◊ < 0.0001<br>♦ <0.0001/+28<br>o <0.0001/+23 |
| ◊ | 3. 6B | N2 | MS(-Glu) | C | 113/117 (3) | 16.45 ± 0.23 | 17 | 20.3 | ♦ 0.0002/+12<br>o < 0.0001 |

| Symbol | Figure | Strain | *B. subtilis* diet | C/U | Deaths/ total (% censored) | Mean±SEM (days) | Median (days) | Max (days)* | *p*-val / % increase ** |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | • 0.0978 |
| ♦ | 3. 6B | N2 | MS(+Glu) | C-U**** | 119/124 (4) | 14.75 ± 0.58 | 13 | 30.8 | ◊ 0.0002<br>o < 0.0001<br>• 0.0002 |
| o | 3. 6B | N2 | Δ*atp*(-Glu) | C | 107/125 (14) | 22.26 ± 0.52 | 21 | 31.2 | ◊ < 0.0001/+35<br>♦ < 0.0001<br>• 0.0016/+24 |
| • | 3. 6B | N2 | Δ*atp*(+Glu) | C-U | 112/121 (7) | 17.97 ± 0.68 | 15 | 34.7 | ◊ 0.0978<br>♦ 0.0002/+22<br>o 0.0016 |

* Maximum lifespan was calculated as the mean of the top 10% longest-lived individuals in each group.

** Where the diet extended lifespan, the percentage increase in mean lifespan (%) is given.

*** L1+Glu: Nematodes were exposed to glucose from L1 onwards, as opposed to L4 onwards.

**** C-U, nematodes were fed on concentrated lawns in the absence of glucose until the L4 stage, at which point they were transferred to unconcentrated lawns in the presence of glucose.

**Table A4 Descriptive statistics and statistical comparisons of square-root-transformed *Phsp-6::gfp* intensity values for *C. elegans* fed on *E. coli* OP50, *B. s.* MS or Δ*atp*.** unconc, unconcentrated; conc, concentrated. Welch ANOVA followed by the Games-Howell *post-hoc* test.

| **Condition** | **N** | **Mean expression** | **St. Dev** | **Test statistic, *p*-value** | ***p*-value (*post-hoc* test)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *E. coli*(-Glu) + PQ | *E. coli*(-Glu) | MS(-Glu) | Δ*atp*(-Glu) | MS(-Glu) conc. | Δ*atp* (-Glu) conc. | *E. coli*(+Glu) | MS(+Glu) |
| *E. coli*(-Glu) + PQ* | 33 | 495.58 | 79.8 | $F(8,122) = 76.47, p < 0.0001$ | - | - | - | - | - | - | - | - |
| *E. coli*(-Glu) | 33 | 321.01 | 77.16 | | < 0.0001 | - | - | - | - | - | - | - |
| *B. s.* MS(-Glu) | 33 | 363.04 | 62.01 | | < 0.0001 | 0.2824 | - | - | - | - | - | - |
| *B. s.* Δ*atp*(-Glu) | 37 | 324.08 | 47.38 | | < 0.0001 | 1 | 0.1029 | - | - | - | - | - |
| *B. s.* MS(-Glu) conc. | 30 | 494 | 59.66 | | 1 | < 0.0001 | < 0.0001 | < 0.0001 | - | - | - | - |
| *B. s.* Δ*atp*(-Glu) conc. | 36 | 461.87 | 78.05 | | 0.7002 | < 0.0001 | < 0.0001 | < 0.0001 | 0.6207 | - | - | - |
| *E. coli*(+Glu) | 39 | 401.51 | 52.35 | | < 0.0001 | 0.0002 | 0.1315 | < 0.0001 | < 0.0001 | 0.0070 | - | - |
| *B. s.* MS(+Glu) | 33 | 359.05 | 67.86 | | < 0.0001 | 0.4652 | 1 | 0.2679 | < 0.0001 | < 0.0001 | 0.1017 | - |
| *B. s.* Δ*atp*(+Glu) | 35 | 264.74 | 33.84 | | < 0.0001 | 0.0104 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |

* Positive control animals were fed on *E. coli* OP50-1(-Glu) and exposed to 200 mM paraquat (PQ) for 15 mins.

**Table A5 Descriptive statistics and statistical comparisons of log-transformed *Pgst-4::gfp* intensity values for *C. elegans* fed on *E. coli* OP50, *B. s.* MS or Δ*atp*.** Welch ANOVA followed by the Games-Howell *post-hoc* test.

| **Condition** | **N** | **Mean expression** | **Standard deviation** | **Test statistic, *p*-value** | ***p*-value (*post-hoc* test)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *E. coli*(-Glu) + PQ | *E. coli*(-Glu) | MS(-Glu) | Δ*atp*(-Glu) | MS(-Glu) conc. | Δ*atp* (-Glu) conc. | *E. coli*(+Glu) | MS(+Glu) |
| *E. coli*(-Glu) + PQ* | 39 | 5.66 | 0.11 | $F(8,122) = 110.84$, $p < 0.0001$ | - | - | - | - | - | - | - | - |
| *E. coli*(-Glu) | 32 | 5.51 | 0.08 | | < 0.0001 | - | - | - | - | - | - | - |
| *B. s.* MS(-Glu) | 34 | 5.573 | 0.11 | | 0.014 | 0.2255 | - | - | - | - | - | - |
| *B. s.* Δ*atp*(-Glu) | 36 | 5.46 | 0.09 | | < 0.0001 | 0.1778 | 0.0003 | - | - | - | - | - |
| *B. s.* MS(-Glu) conc. | 31 | 5.46 | 0.08 | | < 0.0001 | 0.2576 | 0.0005 | 1 | - | - | - | - |
| *B. s.* Δ*atp*(-Glu) conc. | 32 | 5.43 | 0.08 | | < 0.0001 | 0.0051 | < 0.0001 | 0.9311 | 0.8747 | - | - | - |
| *E. coli*(+Glu) | 32 | 5.77 | 0.06 | | 0.0002 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | - | - |
| *B. s.* MS(+Glu) | 32 | 5.95 | 0.14 | | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | - |
| *B. s.* Δ*atp*(+Glu) | 35 | 5.44 | 0.07 | | < 0.0001 | 0.0022 | < 0.0001 | 0.9551 | 0.9041 | 1 | < 0.0001 | < 0.0001 |

* Positive control animals were fed on *E. coli* OP50-1(-Glu) and exposed to 200 mM paraquat (PQ) for 15 mins.

**Table A6 Raw untransformed *Phsp-6::gfp* intensity values for *C. elegans* fed on *E. coli* OP50, *B. s.* MS or Δ*atp*.** The fluorescence values were calculated using the formula: Corrected fluorescence intensity (AU) = Integrated Density – (area of selected nematode x mean fluorescence of 3 background readings).

| Condition | N | Mean expression | Standard deviation | Median |
|---|---|---|---|---|
| *E. coli*(-Glu) + PQ | 33 | 251775.879 | 80369.4299 | 234271 |
| *E. coli*(-Glu) | 33 | 108818.606 | 47088.285 | 109075 |
| *B. s.* MS(-Glu) | 33 | 135523.394 | 46774.4612 | 125616 |
| *B. s.* Δ*atp*(-Glu) | 37 | 107213.081 | 31525.5307 | 97721 |
| *B. s.* MS(-Glu) conc. | 33 | 133381.576 | 51179.7172 | 119481 |
| *B. s.* Δ*atp*(-Glu) conc. | 35 | 71201.0571 | 17692.2332 | 68382 |
| *E. coli*(+Glu) | 39 | 163878.077 | 41697.3439 | 165619 |
| *B. s.* MS(+Glu) | 30 | 247467.167 | 59354.1207 | 240784.5 |
| *B. s.* Δ*atp*(+Glu) | 36 | 219245.472 | 72347.2495 | 220079.5 |

* Positive control animals were fed on *E. coli* OP50-1(-Glu) and exposed to 200 mM paraquat for 15 mins.

**Table A7 Raw untransformed *Pgst-4::gfp* intensity values for *C. elegans* fed on *E. coli* OP50, *B. s*. MS or Δ*atp*.** The fluorescence values were calculated using the formula: Corrected fluorescence intensity (AU) = Integrated Density – (area of selected nematode x mean fluorescence of 3 background readings).

| Condition | N | Mean expression | Standard deviation | Median |
|---|---|---|---|---|
| *E. coli*(-Glu) + PQ | 39 | 474618.462 | 108233.748 | 484904 |
| *E. coli*(-Glu) | 32 | 332064.063 | 62588.3064 | 320759 |
| *B. s.* MS(-Glu) | 34 | 384985.176 | 93419.2114 | 380810 |
| *B. s.* Δ*atp*(-Glu) | 36 | 293991.361 | 59705.7022 | 290647 |
| *B. s.* MS(-Glu) conc. | 31 | 295387.065 | 52972.5109 | 296903 |
| *B. s.* Δ*atp*(-Glu) conc. | 32 | 275685 | 50467.6106 | 276728 |
| *E. coli*(+Glu) | 32 | 588463.156 | 80091.1799 | 611812 |
| *B. s.* MS(+Glu) | 32 | 936545.344 | 302295.898 | 871702 |
| *B. s.* Δ*atp*(+Glu) | 35 | 277026.629 | 41370.4281 | 279293 |

* Positive control animals were fed on *E. coli* OP50-1(-Glu) and exposed to 200 mM paraquat for 15 mins.

**Table A8 Thrashing rate in liquid of *C. elegans* fed on *B. s.* MS or Δ*atp*.** Summary statistics and pairwise comparisons of thrashing rates on the indicated day of adulthood. One-way ANOVA (with Welch's correction for day 1) followed by Tukey's HSD (or Games-Howell for day 1) *post-hoc* test. N = 30.

| **Day** | ***B. s.* diet** | **Mean thrashes ± SD** | **One-way ANOVA Test statistic, *p*-value** | ***Post-hoc* test *p*-value** | | |
|---|---|---|---|---|---|---|
| | | | | MS(-Glu) | Δ*atp*(-Glu) | MS(+Glu) |
| 1 | MS(-Glu) | 76.50 ± 5.73 | $F(3,61) = 70.525$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | 91.93 ± 13.01 | | < 0.0001 | - | - |
| | MS(+Glu) | 96.23 ± 8.74 | | < 0.0001 | 0.44 | - |
| | Δ*atp*(+Glu) | 105.90 ± 11.70 | | < 0.0001 | 0.0003 | 0.0003 |
| 4 | MS(-Glu) | 74.50 ± 9.07 | $F(3,116) = 70.8057$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | 98.17 ± 8.83 | | < 0.0001 | - | - |
| | MS(+Glu) | 78.50 ± 8.17 | | 0.2549 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 93.03 ± 7.29 | | < 0.0001 | 0.0875 | < 0.0001 |
| 7 | MS(-Glu) | 67.10 ± 10.43 | $F(3, 116) = 56.308$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | 99.03 ± 12.44 | | < 0.0001 | - | - |
| | MS(+Glu) | 66.57 ± 12.76 | | 0.9981 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 89.20 ± 11.70 | | < 0.0001 | 0.0092 | < 0.0001 |
| 10 | MS(-Glu) | 58.10 ± 10.14 | $F(3, 116) = 87.868$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | 96.23 ± 10.53 | | < 0.0001 | - | - |
| | MS(+Glu) | 61.60 ± 11.93 | | 0.5689 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 82.07 ± 9.13 | | < 0.0001 | < 0.0001 | < 0.0001 |

**Appendix B**

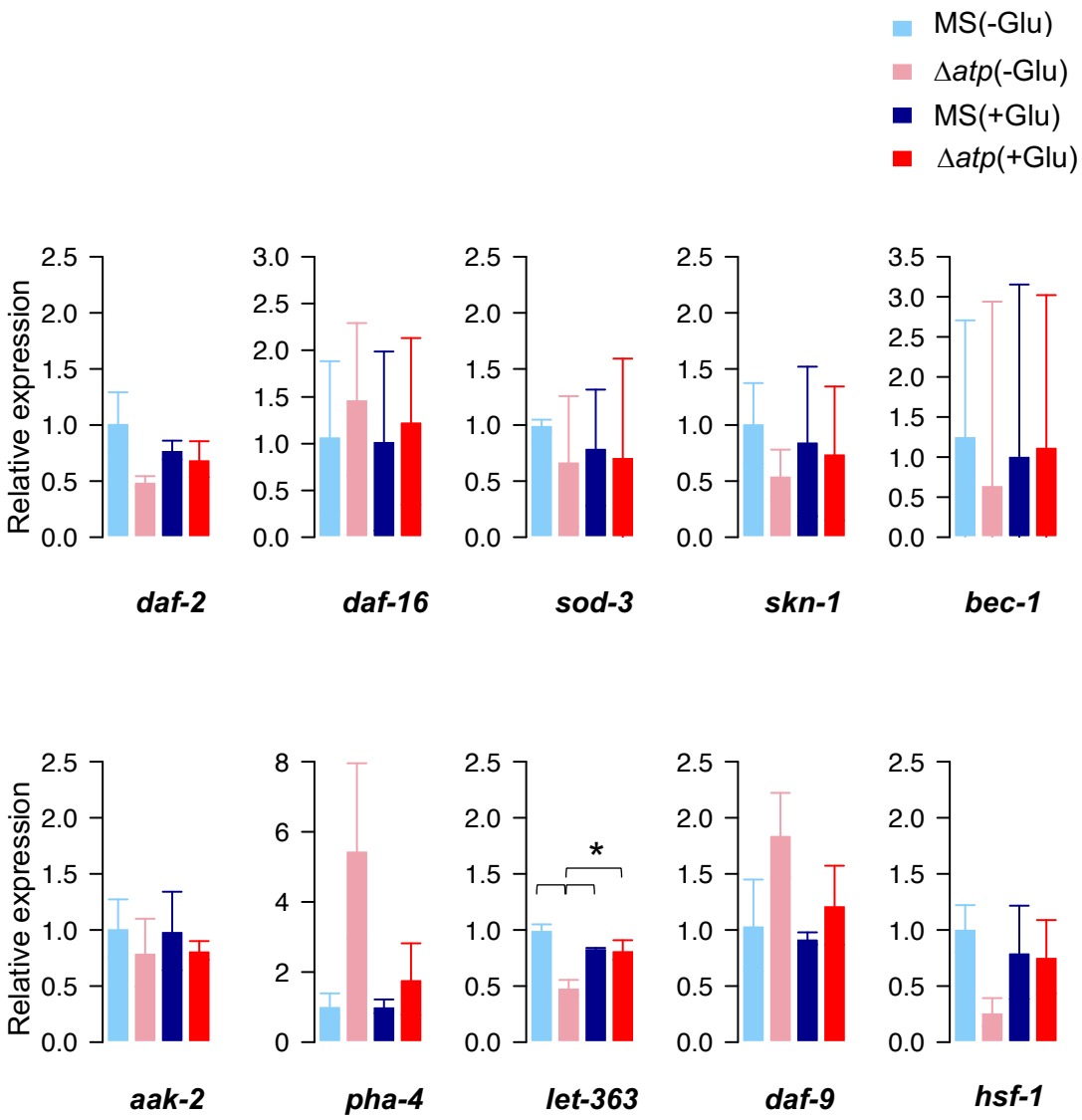



**Figure B1 qRT-PCR expression analysis of genes involved in IIS signalling, TOR signalling and DR.** Expression was measured in day 2 adult *C. elegans* raised on each of the *B. subtilis* diets and expressed as 2^-ΔΔCt values relative to *B. s.* MS(-Glu) (N = 2 for each cohort). Animals fed on *B. s.* Δ*atp*(-Glu) displayed a downregulation of *let-363*/TOR expression. Bars show mean ± standard deviation of relative expression. ΔCt values were calculated relative to *act-1* expression and were compared using ANOVA and Tukey's *post-hoc* test. *, $p < 0.05$.

**Table B1 Descriptive statistics and within-day statistical comparisons of body volume data for *C. elegans* fed on *B. s.* MS or Δ*atp*.** Kruskal-Wallis test with Dunn's *post-hoc* test (FDR correction). St. Dev., standard deviation. IQR, interquartile range. Mean ranks were calculated from the Kruskal-Wallis test.

| Day | Condition | N | Mean vol., pL | St. Dev. | Median vol., pL | IQR | Mean Rank | Test statistic, *p*-value | *p*-value (*post-hoc* test) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | MS(-Glu) | Δ*atp*(-Glu) | MS(+Glu) |
| 1 | MS(-Glu) | 73 | 5271.90 | 767.75 | 5215.036 | 1101.97 | 167.59 | $\chi2$ = 141.24, *p* < 0.0001 | - | - | - |
| | Δ*atp*(-Glu) | 93 | 5546.44 | 700.65 | 5425.43 | 750.99 | 189.19 | | 0.078 | - | - |
| | MS(+Glu) | 57 | 3820.29 | 545.82 | 3775.53 | 697.57 | 51.26 | | < 0.0001 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 48 | 4228.03 | 915.48 | 4278.92 | 906.86 | 85.52 | | < 0.0001 | < 0.0001 | 0.031 |
| 3 | MS(-Glu) | 79 | 7890.24 | 658.78 | 7821.85 | 1016.94 | 160.96 | $\chi2$ = 230.22, *p* < 0.0001 | - | - | - |
| | Δ*atp*(-Glu) | 78 | 9449.95 | 1062.05 | 9509.63 | 1493.02 | 246.87 | | < 0.0001 | - | - |
| | MS(+Glu) | 59 | 7752.95 | 651.61 | 7783.00 | 679.37 | 151.42 | | 0.52 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 81 | 5009.90 | 772.96 | 4982.46 | 1194.85 | 41.32 | | < 0.0001 | < 0.0001 | < 0.0001 |
| 5 | MS(-Glu) | 73 | 10911.16 | 1705.38 | 10395.63 | 2105.42 | 246.60 | $\chi2$ = 270.53, *p* < 0.0001 | - | - | - |
| | Δ*atp*(-Glu) | 76 | 10654.69 | 1377.09 | 10319.65 | 1309.45 | 243.86 | | 0.86 | - | - |
| | MS(+Glu) | 81 | 7577.72 | 78 | 7574.02 | 1078.48 | 131.94 | | < 0.0001 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 90 | 4992.10 | 628.09 | 4942.26 | 843.76 | 45.98 | | < 0.0001 | < 0.0001 | < 0.0001 |
| 7 | MS(-Glu) | 78 | 11329.05 | 1372.61 | 11428.75 | 1884.75 | 200.08 | $\chi2$ = 263.77, *p* < 0.0001 | - | - | - |
| | Δ*atp*(-Glu) | 82 | 13293.06 | 1539.20 | 13101.01 | 1709.76 | 257.26 | | < 0.0001 | - | - |
| | MS(+Glu) | 75 | 8603.91 | 1008.6 | 8718.60 | 1175.76 | 117.33 | | < 0.0001 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 76 | 5665.15 | 769.81 | 5605.27 | 943.95 | 38.50 | | < 0.0001 | < 0.0001 | < 0.0001 |

| Day | Condition | N | Mean vol., pL | St. Dev. | Median vol., pL | IQR | Mean Rank | Test statistic, *p*-value | *p*-value (*post-hoc* test) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | MS(-Glu) | Δ*atp*(-Glu) | MS(+Glu) |
| 9 | MS(-Glu) | 82 | 10808.443 | 1249.228 | 10815.23 | 1177.48 | 227.99 | $\chi 2$ = 258.48, $p$ < 0.0001 | - | - | - |
| | Δ*atp*(-Glu) | 74 | 12122.386 | 1876.608 | 11814.61 | 1914.75 | 267.28 | | 0.0098 | - | - |
| | MS(+Glu) | 87 | 8461.36 | 1029.404 | 8717.82 | 1342.90 | 128.28 | | < 0.0001 | < 0.0001 | - |
| | Δ*atp*(+Glu) | 85 | 6608.353 | 837.277 | 6444.03 | 1195.74 | 50.85 | | < 0.0001 | < 0.0001 | < 0.0001 |

**Table B2 Number of viable progeny laid per day of adulthood (1-6) by *C. elegans* fed on *B. s.* MS or Δ*atp***. IQR, interquartile range.

| Day | Condition | N | Mean progeny | St. Dev. | Median progeny | IQR |
|---|---|---|---|---|---|---|
| **1** | MS(-Glu) | 17 | 48.65 | 10.01 | 51.0 | 13 |
| | MS(+Glu) | 18 | 38.78 | 10.51 | 37.5 | 15.75 |
| | Δ*atp*(-Glu) | 19 | 33.63 | 10.10 | 33.0 | 12.5 |
| | Δ*atp*(+Glu) | 21 | 14.57 | 6.59 | 16.0 | 10 |
| **2** | MS(-Glu) | 17 | 127.59 | 11.06 | 130.0 | 7 |
| | MS(+Glu) | 19 | 103.05 | 11.31 | 101.0 | 11.5 |
| | Δ*atp*(-Glu) | 19 | 107.89 | 18.13 | 112.0 | 21 |
| | Δ*atp*(+Glu) | 19 | 44.47 | 12.68 | 43.0 | 13 |
| **3** | MS(-Glu) | 16 | 76.13 | 19.38 | 82.5 | 16.25 |
| | MS(+Glu) | 17 | 90.71 | 24.88 | 97.0 | 39 |
| | Δ*atp*(-Glu) | 18 | 57.17 | 21.22 | 57.5 | 29.5 |
| | Δ*atp*(+Glu) | 19 | 43.84 | 14.00 | 44.0 | 22 |
| **4** | MS(-Glu) | 15 | 2.60 | 4.39 | 1.0 | 2 |
| | MS(+Glu) | 19 | 9.68 | 8.96 | 7.0 | 9.5 |
| | Δ*atp*(-Glu) | 18 | 8.11 | 10.13 | 2.5 | 9.75 |
| | Δ*atp*(+Glu) | 18 | 29.44 | 10.02 | 26.5 | 11.75 |
| **5** | MS(-Glu) | 14 | 0.21 | 0.43 | 0.0 | 0 |
| | MS(+Glu) | 19 | 0.95 | 3.21 | 0.0 | 0 |
| | Δ*atp*(-Glu) | 18 | 1.78 | 5.55 | 0.0 | 0 |
| | Δ*atp*(+Glu) | 11 | 21.73 | 9.23 | 23.0 | 11.5 |
| **6** | MS(-Glu) | 15 | 0.00 | 0.00 | 0.0 | 0 |
| | MS(+Glu) | 19 | 0.00 | 0.00 | 0.0 | 0 |
| | Δ*atp*(-Glu) | 18 | 0.00 | 0.00 | 0.0 | 0 |
| | Δ*atp*(+Glu) | 5 | 15.60 | 5.37 | 13.0 | 2 |

**Table B3 Statistical comparisons of daily progeny production of *C. elegans* fed on *B. s.* MS or Δ*atp*.** One-way ANOVA followed by Tukey's HSD *post-hoc* test (day 1 and 3) or Kruskal-Wallis test followed by Dunn's *post-hoc* test with FDR *p*-value correction (day 2 and 4). Mean rank values were calculated using the Kruskal-Wallis test (days 2 and 4).

| **Day** | ***B. s.* diet** | **Mean Rank** | **Test statistic, *p*-value** | ***Post-hoc* test *p*-value** | | |
|---|---|---|---|---|---|---|
| | | | | MS(-Glu) | Δ*atp*(-Glu) | MS(+Glu) |
| **1** | MS(-Glu) | - | $F(3,71) = 45.517$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | - | | < 0.0001 | - | - |
| | MS(+Glu) | - | | 0.0134 | 0.344 | - |
| | Δ*atp*(+Glu) | - | | < 0.0001 | < 0.0001 | < 0.0001 |
| **2** | MS(-Glu) | 61.62 | $\chi2 = 53.823$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | 43.61 | | 0.0145 | - | - |
| | MS(+Glu) | 37.24 | | 0.001 | 0.3613 | - |
| | Δ*atp*(+Glu) | 10.08 | | < 0.0001 | < 0.0001 | 0.0002 |
| **3** | MS(-Glu) | - | $F(3,66) = 18.732$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | - | | 0.0383 | - | - |
| | MS(+Glu) | - | | 0.1702 | < 0.0001 | - |
| | Δ*atp*(+Glu) | - | | < 0.0001 | 0.1935 | < 0.0001 |
| **4** | MS(-Glu) | 18.30 | $\chi2 = 35.653$, $p < 0.0001$ | - | - | - |
| | Δ*atp*(-Glu) | 28.97 | | 0.1586 | - | - |
| | MS(+Glu) | 33.63 | | 0.0429 | 0.4847 | - |
| | Δ*atp*(+Glu) | 58.33 | | < 0.0001 | < 0.0001 | 0.00042 |

**Table B4 Brood size of *C. elegans* fed on *B. s.* MS or Δ*atp*.** Total brood size of animals fed on *B. s.* Δ*atp*(+Glu) could not be determined as a result of nematode escape. One-way ANOVA followed by Tukey's *post-hoc* test.

| **Condition** | **Mean** | **St. Dev.** | **One-way ANOVA** | | **Tukey's *post-hoc* test *p* value** | | |
|---|---|---|---|---|---|---|---|
| | | | *F(2,27)* | *p* | MS (-Glu) | Δ*atp* (-Glu) | MS (+Glu) |
| MS(-Glu) | 248.27 | 35.17 | 5.915 | 0.0052 | - | 0.005 | 0.5869 |
| *Δatp*(-Glu) | 210.72 | 20.87 | | | 0.005 | - | 0.0602 |
| MS(+Glu) | 236.75 | 25.72 | | | 0.5869 | 0.060 | - |

**Table B5 Width of *B. subtilis* MS and Δ*atp* cells.** The width of cells from concentrated lawns in the absence of glucose was also measured. Kruskal-Wallis H statistic = 248.49, *p* value < 0.0001. Multiple comparisons were conducted using Dunn's *post-hoc* test (FDR correction).

| **Diet** | **Mean width** | **St. Dev.** | **Median width** | **IQR** | **Mean Rank** | ***Post-hoc* test *p* value** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **MS(-Glu)** | **Δ*atp*(-Glu)** | **MS(+Glu)** | **Δ*atp*(+Glu)** | **MS(-Glu) conc.** | **Δ*atp*(-Glu) conc.** |
| MS(-Glu) | 0.981 | 0.144 | 0.979 | 0.225 | 135.34 | - | - | - | - | - | - |
| Δ*atp*(-Glu) | 1.301 | 0.170 | 1.274 | 0.2545 | 449.61 | < 0.0001 | - | - | - | - | - |
| MS(+Glu) | 1.134 | 0.115 | 1.130 | 0.136 | 286.47 | < 0.0001 | < 0.0001 | - | - | - | - |
| Δ*atp*(+Glu) | 1.265 | 0.159 | 1.250 | 0.192 | 419.46 | < 0.0001 | 0.2232 | < 0.0001 | - | - | - |
| MS(-Glu) conc. | 1.063 | 0.097 | 1.070 | 0.13 | 199.5 | 0.0110 | < 0.0001 | 0.0006 | | - | - |
| Δ*atp*(-Glu) conc. | 1.177 | 0.110 | 1.187 | 0.141 | 348.62 | < 0.0001 | < 0.0001 | < 0.0001 | 0.0053 | < 0.0001 | - |

**Table B6 Length of *B. subtilis* MS and Δ*atp* cells.** The length of cells from concentrated lawns in the absence of glucose was also measured. Kruskal-Wallis H statistic = 666.16, *p* value < 0.0001. Multiple comparisons were conducted using Dunn's *post-hoc* test (FDR correction).

| **Diet** | **Mean length** | **St. Dev.** | **Median length** | **IQR** | **Mean Rank** | ***Post-hoc* test *p* value** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **MS(-Glu)** | **Δ*atp*(-Glu)** | **MS(+Glu)** | **Δ*atp*(+Glu)** | **MS(-Glu) conc.** | **Δ*atp*(-Glu) conc.** |
| MS(-Glu) | 3.11 | 0.53 | 3.02 | 0.627 | 239.88 | - | - | - | - | - | - |
| Δ*atp*(-Glu) | 4.60 | 1.33 | 4.43 | 1.815 | 602.48 | < 0.0001 | - | - | - | - | - |
| MS(+Glu) | 6.22 | 2.06 | 5.90 | 2.1155 | 833.32 | < 0.0001 | < 0.0001 | - | - | - | - |
| Δ*atp*(+Glu) | 6.62 | 1.97 | 6.34 | 2.71 | 870.35 | < 0.0001 | < 0.0001 | 0.26 | - | - | - |
| MS(-Glu) conc. | 3.23 | 0.53 | 3.21 | 0.674 | 280.03 | 0.24 | < 0.0001 | < 0.0001 | < 0.0001 | - | - |
| Δ*atp*(-Glu) conc. | 3.86 | 0.87 | 3.78 | 1.244 | 452.95 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | | - |

**Table B7 Descriptive statistics and comparative analysis of pharyngeal pumping rates min$^{-1}$ of *C. elegans* fed on *B. s.* MS or Δ*atp*.** Significance was determined using Kruskal-Wallis test followed by Dunn's *post-hoc* test. IQR, interquartile range. Mean ranks were calculated from the Kruskal-Wallis test.

| Day | Condition | N | Mean pumping | St. Dev. | Median pumping | IQR | Mean Rank | Test statistic, *p*-value | *Post-hoc* test *p* value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | MS(-Glu) | Δ*atp*(-Glu) | MS(+Glu) |
| 1 | MS(-Glu) | 21 | 282.76 | 23.58 | 287 | 26 | 54.79 | $\chi2(3) =$ 17.982 $p =$ 0.0004 | - | - | - |
| | Δ*atp*(-Glu) | 24 | 310.13 | 8.86 | 312 | 14 | 74.542 | | 0.0002 | - | - |
| | MS(+Glu) | 21 | 299.52 | 22.89 | 301 | 28 | 78.514 | | 0.0133 | 0.2145 | - |
| | Δ*atp*(+Glu) | 22 | 297.05 | 32.97 | 307 | 18.75 | 82.153 | | 0.0133 | 0.2145 | 0.9802 |
| 4 | MS(-Glu) | 22 | 280.09 | 17.14 | 280.50 | 24.75 | 50.069 | $\chi2(3) =$ 34.567 $p <$ 0.0001 | - | - | - |
| | Δ*atp*(-Glu) | 24 | 311.33 | 12.69 | 314.50 | 16.75 | 79.208 | | < 0.0001 | - | - |
| | MS(+Glu) | 20 | 297.70 | 13.76 | 301.50 | 12.25 | 78.056 | | 0.0119 | 0.2145 | - |
| | Δ*atp*(+Glu) | 23 | 302 | 17.04 | 309 | 12 | 82.667 | | 0.0002 | 0.1112 | 0.2197 |
| 7 | MS(-Glu) | 36 | 230.69 | 79.59 | 257 | 66.25 | 37.347 | $\chi2(3) =$ 32.727, $p <$ 0.0001 | - | - | - |
| | Δ*atp*(-Glu) | 28 | 294.14 | 18.77 | 300 | 18.75 | 93.167 | | < 0.0001 | - | - |
| | MS(+Glu) | 29 | 285.86 | 22.72 | 293 | 29 | 83.431 | | 0.0002 | 0.1747 | - |
| | Δ*atp*(+Glu) | 27 | 273.63 | 21.76 | 272 | 28.50 | 76.056 | | 0.0742 | 0.0034 | 0.0742 |
| 10 | MS(-Glu) | 20 | 162.50 | 99.78 | 167.50 | 150.5 | 52.889 | $\chi2(3) =$ 20.174, $p =$ 0.0002 | - | - | - |
| | Δ*atp*(-Glu) | 20 | 255.35 | 67.47 | 278 | 16.25 | 75.986 | | 0.0006 | - | - |
| | MS(+Glu) | 20 | 208.55 | 110.04 | 269 | 66.75 | 74.278 | | 0.0994 | 0.0656 | - |
| | Δ*atp*(+Glu) | 21 | 254.95 | 71.71 | 278 | 25 | 86.847 | | 0.0006 | 0.8541 | 0.0721 |

**Table B8 Percentages of *C. elegans* on day 1, 4, 7, 10 and 14 of adulthood in each zone of the agar plate seeded with either *B. s.* MS or Δ*atp*.** N = 40 for each condition per day. Data were collected by Neilson (2017).

| **Day 1** | Diet | | | |
|---|---|---|---|---|
| **Zone** | *B. s.* MS(+Glu) | *B. s. Δatp*(+Glu) | *B. s.* MS(-Glu) | *B. s. Δatp*(-Glu) |
| **1** | 88 | 97 | 89 | 97 |
| **2** | 12 | 0 | 3 | 0 |
| **3** | 0 | 0 | 0 | 0 |
| **4** | 0 | 3 | 8 | 3 |
| **Day 4** | Diet | | | |
| **Zone** | *B. s.* MS(+Glu) | *B. s. Δatp*(+Glu) | *B. s.* MS(-Glu) | *B. s. Δatp*(-Glu) |
| **1** | 30 | 22 | 98 | 78 |
| **2** | 1 | 35 | 0 | 2 |
| **3** | 42 | 40 | 0 | 0 |
| **4** | 27 | 3 | 2 | 20 |
| **Day 7** | Diet | | | |
| **Zone** | *B. s.* MS(+Glu) | *B. s. Δatp*(+Glu) | *B. s.* MS(-Glu) | *B. s. Δatp*(-Glu) |
| **1** | 42 | 14 | 39 | 64 |
| **2** | 20 | 0 | 0 | 0 |
| **3** | 36 | 76 | 59 | 36 |
| **4** | 2 | 10 | 2 | 0 |
| **Day 10** | Diet | | | |
| **Zone** | *B. s.* MS(+Glu) | *B. s. Δatp*(+Glu) | *B. s.* MS(-Glu) | *B. s. Δatp*(-Glu) |
| **1** | 60 | 8 | 99 | 89 |
| **2** | 0 | 1 | 0 | 2 |
| **3** | 39 | 88 | 0 | 9 |
| **4** | 1 | 3 | 1 | 0 |
| **Day 14** | Diet | | | |
| **Zone** | *B. s.* MS(+Glu) | *B. s. Δatp*(+Glu) | *B. s.* MS(-Glu) | *B. s. Δatp*(-Glu) |
| **1** | 26 | 4 | 77 | 92 |
| **2** | 7 | 0 | 22 | 0 |
| **3** | 65 | 88 | 1 | 0 |
| **4** | 2 | 8 | 0 | 8 |

**Table B9 Statistical comparison of the association between lawn location and diet (*B. s.* MS or Δ*atp*).** Data in Table B8 were analysed using the Chi-Square test.

| Day of adulthood | Test statistic | *p*-value |
|---|---|---|
| 1 | 0.017 | 0.896 |
| 4 | 70.524 | < 0.0001 |
| 7 | 9.119 | 0.003 |
| 10 | 75.456 | < 0.0001 |
| 14 | 79.349 | < 0.0001 |

**Table B10 Summary of lifespan assays in the presence of 0.7 μg/ml chloramphenicol.** The figure panels to which the experiment refers are indicated. The mean and median lifespan and standard error values were calculated from a log rank test from samples of 114-129 individuals each. (-Glu), minus glucose; (+Glu), plus glucose 2%. l, lost through escape; b, bagged; v, vulval explosion. Symbols indicate comparisons between groups. *p*, log-rank test on Kaplan-Meier survival curves with Holm-Sidak multiple comparison *p*-value correction. ns, no significant difference ($p > 0.05$).

| Symbol | Figure | Strain | *B. subtilis* diet | C/U | Deaths/ total (% censored) | Censored | Mean±SEM (days) | Median (days) | Max*(days) | *p*-val / % increase** |
|---|---|---|---|---|---|---|---|---|---|---|
| ◊ | 4. 10 | N2 | MS(-Glu) | U | 106/127 (17) | 21 (20v;1b) | 18.1 ± 0.4 | 17 ± 0.3 | 25.4 | ♦ < 0.0001 / +12<br>o < 0.0001 / +13<br>• 0.00344 / +10 |
| ♦ | 4. 10 | N2 | MS(+Glu) | U | 115/129 (11) | 14(11v;3b) | 16.1 ± 0.3 | 16 ± 0.2 | 22.5 | ◊ < 0.0001<br>o < 0.0001<br>• 0.3367 (ns) |
| o | 4. 10 | N2 | Δ*atp*(-Glu) | U | 95/114 (17) | 19(19v) | 20.5 ± 0.3 | 20 ± 0.3 | 25.5 | ◊ 0.0007 / +13<br>♦ < 0.0001 / +27<br>• < 0.0001 / +24 |
| • | 4. 10 | N2 | Δ*atp*(+Glu) | U | 126/129 (2) | 3(1v;2b) | 16.5 ± 0.3 | 16 ± 0.3 | 23 | ◊ 0.0034<br>♦ 0.3367 (ns)<br>o < 0.0001 |

* Maximum lifespan was calculated as the mean of the top 10% longest-lived individuals in each group.

** Where the diet extended lifespan, the percentage increase in mean lifespan (%) is given.

**Table B11 Pharyngeal pumping min$^{-1}$ of *eat-2(ad1116)* mutant *C. elegans* fed on *B. s.* MS(+Glu) or Δ*atp*(+Glu).** N = 10 per condition.

| Condition | MS (+Glu) | Δ*atp* (+Glu) | MS (+Glu) | Δ*atp* (+Glu) | MS (+Glu) | Δ*atp* (+Glu) | MS (+Glu) | Δ*atp* (+Glu) |
|---|---|---|---|---|---|---|---|---|
| **Day** | **1** | | **4** | | **7** | | **10** | |
| Mean | 36.8 | 38.8 | 38.4 | 36.8 | 38.8 | 28.9 | 33.3 | 24.7 |
| St. Dev | 14.62 | 14.80 | 6.65 | 17.27 | 19.41 | 16.25 | 10.16 | 18.88 |
| Median | 37 | 46.5 | 41 | 36 | 44 | 29.5 | 30.5 | 22.5 |

**Table B12 Statistical comparison of pharyngeal pumping in *eat-2(ad1116)* individuals fed on *B. s.* MS(+Glu) or Δ*atp*(+Glu).** See Table B11 for summary data**.** Sample differences were analysed per day with a one-sample t test without (days 1, and 7) or with (days 4 and 10) Welch's correction for unequal variance.

| Day of adulthood | One-sample t statistic (df) | *p* value |
|---|---|---|
| 1 | 0.304 (18) | 0.7646 |
| 4 | -0.2734 (11.615) | 0.7893 |
| 7 | -1.2368 (18) | 0.2321 |
| 10 | -1.2686 (13.805) | 0.2256 |

**Table B13 Summary of lifespan assays of N2 and *eat-2(ad1116)* in the presence of glucose.** The figure panels to which the experiment refers are indicated. The mean and median lifespan and standard error values were calculated from a log rank test from samples of at least 100 individuals each. (-Glu), minus glucose; (+Glu), plus glucose 2%. C, concentrated *B. subtilis* lawn. U, un-concentrated lawn. l, lost through escape; b, bagged; v, vulval explosion. Symbols indicate comparisons between groups. *p*, log-rank test on Kaplan-Meier survival curves with Holm-Sidak multiple comparison *p* -value correction. ns, no significant difference ($p > 0.05$).

| Symbol | Figure | Strain | *B. subtilis* diet | C/U | Deaths/ total (% censored) | Censored | Mean±SEM (days) | Median (days) | Max* (days) | *p*-val / % increase in mean lifespan ** |
|---|---|---|---|---|---|---|---|---|---|---|
| ♦ | 4. 11 | N2 | MS(+Glu) | C-U*** | 119/126 (6) | 7 (3b; 4l) | 18.2±0.4 | 18±0.5 | 24.4 | • < 0.0001<br>o < 0.0001<br>◊ < 0.0001 |
| • | 4. 11 | N2 | Δ*atp*(+Glu) | C-U | 122/130 (7) | 8 (2b; 6l) | 21.7±0.4 | 21±0.63 | 29.4 | ♦ < 0.0001/+19<br>o < 0.0001<br>◊ < 0.0001 |
| o | 4. 11 | *eat-2(ad1116)* | MS(+Glu) | C-U | 119/136 (14) | 17 (14b; 3l) | 24.9±0.5 | 26±0.37 | 30.8 | ♦ < 0.0001/+37<br>• < 0.0001 / +15<br>◊ < 0.0001 |
| ◊ | 4. 11 | *eat-2(ad1116)* | Δ*atp*(+Glu) | C-U | 118/144 (22) | 26 (7b;1v;18l) | 30±0.6 | 30±0.6 | 37 | ♦ < 0.0001<br>• < 0.0001/+35<br>o < 0.0001/+17 |

* Maximum lifespan was calculated as the mean of the top 10% longest-lived individuals in each group.

** Where the diet extended lifespan, the percentage increase in mean lifespan (%) is given.

*** C-U, nematodes were fed on concentrated lawns in the absence of glucose until the L4 stage, at which point they were transferred to unconcentrated lawns in the presence of glucose.

**Table B14 Raw data for qRT-PCR analysis of *C. elegans* gene expression.** ΔCt values were calculated with two internal controls (shaded cells = *pmp-3*, unshaded cells = *act-1*) for two independent biological replicates. ΔΔCt values were calculated by subtracting the average ΔCt value of the corresponding gene expressed in *B. s.* MS(-Glu) from each ΔCt value. The relative abundance (2^(-ΔΔCt)) of each gene was derived from this, and the average and standard deviation (St. Dev.) of these values are given.

| Gene | Bio. rep. | ΔCt | | | | Average abundance | | | | St. Dev. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | -Glu | | +Glu | | -Glu | | +Glu | | -Glu | | +Glu | |
| | | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* |
| *daf-16* | 1 | 2.41 | 0.97 | 2.45 | 1.83 | 1.03 | 2.61 | 1.16 | 1.63 | 0.34 | 1.17 | 0.1 | 0.37 |
| | 2 | 3.09 | 1.91 | 2.62 | 2.30 | | | | | | | | |
| | 1 | 5.72 | 5.26 | 5.72 | 5.48 | 1.08 | 1.47 | 1.03 | 1.24 | 0.57 | 0.79 | 0.64 | 0.72 |
| | 2 | 6.85 | 6.41 | 7.07 | 6.74 | | | | | | | | |
| *sod-3* | 1 | 4.80 | 4.00 | 4.81 | 4.42 | 1.02 | 1.2 | 0.98 | 0.94 | 0.26 | 0.36 | 0.21 | 0.21 |
| | 2 | 4.28 | 4.61 | 4.36 | 4.86 | | | | | | | | |
| | 1 | 8.11 | 8.29 | 8.08 | 8.07 | 1 | 0.67 | 0.8 | 0.71 | 0.03 | 0.27 | 0.28 | 0.41 |
| | 2 | 8.04 | 9.11 | 8.82 | 9.31 | | | | | | | | |
| *skn-1* | 1 | -1.58 | -1.60 | -1.46 | -1.61 | 1 | 0.99 | 1.02 | 1.02 | 0.03 | 0.06 | 0.12 | 0.03 |
| | 2 | -1.53 | -1.48 | -1.70 | -1.56 | | | | | | | | |
| | 1 | 1.73 | 2.69 | 1.81 | 2.04 | 1.02 | 0.55 | 0.85 | 0.75 | 0.25 | 0.09 | 0.38 | 0.3 |
| | 2 | 2.23 | 3.02 | 2.76 | 2.88 | | | | | | | | |

| Gene | Bio. rep. | ΔCt | | | | Average abundance | | | | St. Dev. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | -Glu | | +Glu | | -Glu | | +Glu | | -Glu | | +Glu | |
| | | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* |
| *bec-1* | 1 | -0.11 | -0.30 | 0.10 | -0.39 | 1.16 | 1.12 | 0.96 | 1.35 | 0.82 | 1.23 | 0.77 | 1.09 |
| | 2 | 1.48 | 2.73 | 1.94 | 1.49 | | | | | | | | |
| | 1 | 3.20 | 3.99 | 3.37 | 3.26 | 1.26 | 0.65 | 1.02 | 1.13 | 1.09 | 0.74 | 1.12 | 1.16 |
| | 2 | 5.25 | 7.23 | 6.39 | 5.94 | | | | | | | | |
| *pha-4* | 1 | 5.58 | 2.35 | 5.39 | 4.83 | 1.01 | 8.86 | 1.21 | 2.47 | 0.2 | 1.03 | 0.31 | 1.43 |
| | 2 | 5.16 | 2.11 | 4.85 | 3.57 | | | | | | | | |
| | 1 | 9.15 | 6.90 | 8.97 | 8.68 | 1.03 | 5.47 | 1.02 | 1.8 | 0.36 | 2.49 | 0.2 | 1.02 |
| | 2 | 8.42 | 5.93 | 8.57 | 7.45 | | | | | | | | |
| *aak-2* | 1 | 1.65 | 1.27 | 1.70 | 1.56 | 1 | 1.28 | 1.22 | 1.1 | 0.14 | 0.14 | 0.49 | 0.2 |
| | 2 | 1.36 | 1.04 | 0.86 | 1.19 | | | | | | | | |
| | 1 | 5.15 | 5.66 | 5.31 | 5.28 | 1.02 | 0.8 | 0.99 | 0.82 | 0.26 | 0.3 | 0.35 | 0.08 |
| | 2 | 4.63 | 4.87 | 4.58 | 5.08 | | | | | | | | |
| *daf-2* | 1 | -0.50 | -0.55 | -0.41 | -0.22 | 1.01 | 0.82 | 0.95 | 0.94 | 0.16 | 0.15 | 0.15 | 0.29 |
| | 2 | -0.82 | -0.19 | -0.74 | -0.86 | | | | | | | | |
| | 1 | 3.00 | 3.84 | 3.20 | 3.50 | 1.02 | 0.49 | 0.78 | 0.69 | 0.27 | 0.05 | 0.08 | 0.16 |
| | 2 | 2.44 | 3.64 | 2.98 | 3.03 | | | | | | | | |
| *mtl-1* | 1 | -0.99 | -1.67 | -0.59 | -0.44 | 1 | 1.12 | 1.37 | 0.66 | 0.01 | 0.66 | 0.88 | 0.03 |
| | 2 | -1.00 | -0.38 | -0.02 | -0.36 | | | | | | | | |
| | 1 | 2.58 | 2.89 | 2.99 | 3.41 | 1 | 0.61 | 0.56 | 0.49 | 0.04 | 0.32 | 0.31 | 0.13 |

| Gene | Bio. rep. | ΔCt | | | | Average abundance | | | | St. Dev. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | -Glu | | +Glu | | -Glu | | +Glu | | -Glu | | +Glu | |
| | | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* | MS | Δ*atp* |
| | 2 | 2.67 | 3.98 | 4.20 | 3.97 | | | | | | | | |
| *hsp-70* | 1 | -1.66 | - | 0.06 | -0.36 | 1 | 1.48 | 1.6 | 0.55 | 0.13 | | 1.79 | 0.14 |
| | 2 | -1.38 | -0.50 | -1.13 | -0.90 | | | | | | -0.5 | | |
| | 1 | 1.92 | - | 3.64 | 3.49 | 1.01 | 0.29 | 0.42 | 0.39 | 0.18 | | 0.11 | 0.01 |
| | 2 | 2.29 | 3.87 | 3.09 | 3.43 | | | | | | 3.867 | | |
| *daf-9* | 1 | 8.63 | 6.25 | 8.28 | 7.44 | 1.06 | 3.21 | 0.45 | 1.62 | 0.5 | 0.7 | 0.64 | 0.01 |
| | 2 | 7.64 | 6.69 | 7.55 | 7.43 | | | | | | | | |
| | 1 | 12.13 | 10.64 | 11.89 | 11.16 | 1.04 | 1.85 | 0.92 | 1.22 | 0.41 | 0.37 | 0.05 | 0.35 |
| | 2 | 11.31 | 11.06 | 11.77 | 11.76 | | | | | | | | |
| *let-363* | 1 | 0.10 | 0.16 | 0.29 | 0.11 | 1 | 0.85 | 0.9 | 1.12 | 0.04 | 0.13 | 0.07 | 0.21 |
| | 2 | 0.03 | 0.47 | -0.30 | -0.29 | | | | | | | | |
| | 1 | 3.60 | 4.55 | 3.90 | 3.83 | 1 | 0.49 | 0.83 | 0.82 | 0.05 | 0.07 | 0.01 | 0.09 |
| | 2 | 3.70 | 4.84 | 3.93 | 4.05 | | | | | | | | |
| *hsf-1* | 1 | -1.05 | -0.32 | -0.97 | -1.10 | 1.01 | 0.63 | 1.45 | 1.01 | 0.16 | 0.06 | 0.55 | 0.21 |
| | 2 | -0.72 | 0.88 | -0.50 | -0.67 | | | | | | | | |
| | 1 | 2.53 | 4.23 | 2.61 | 2.75 | 1.01 | 0.27 | 0.8 | 0.76 | 0.21 | 0.13 | 0.42 | 0.33 |
| | 2 | 2.95 | 5.25 | 3.72 | 3.66 | | | | | | | | |

**Table B15 Statistical comparison of qRT-PCR ΔCt expression values from *C. elegans* fed on *B. s.* MS or Δ*atp*.** See Table B14 for raw data. One-way ANOVA (num df = 3, denom df =4) was used to find differences in expression. Tukey's *post-hoc* test was used to find pairwise difference if ANOVA $p < 0.05$. ΔCt values were calculated in comparison to *pmp-3* or *act-1*. Asterisks indicate the condition with higher expression in significant comparisons.

| Internal control | Gene | Test statistic | *P* value | Tukey's *post-hoc* test (t statistic, *p* value) (df = 4) |
|---|---|---|---|---|
| *pmp-3* | *daf-2* | 0.3152 | 0.8149 | |
| | *daf-16* | 3.3348 | 0.1375 | |
| | *sod-3* | 0.3305 | 0.8051 | |
| | *skn-1* | 0.0890 | 0.9623 | |
| | *bec-1* | 0.0786 | 0.9683 | |
| | *aak-2* | 0.3601 | 0.7862 | |
| | *pha-4* | 15.49 | 0.0115 | Δ*atp*(-Glu) v Δ*atp*(+Glu): 3.8514, 0.0596<br>Δ*atp*(-Glu)* v MS(-Glu): 6.1303, 0.0123<br>Δ*atp*(-Glu)* v MS(+Glu): 5.6459, 0.0165<br>Δ*atp*(+Glu) v MS(-Glu): 2.279, 0.2458<br>Δ*atp*(+Glu) v MS(+Glu): 1.7946, 0.3916<br>MS(-Glu) v MS(+Glu): 0.4845, 0.9586 |
| | *let-363* | 0.7858 | 0.5611 | |
| | *daf-9* | 5.0728 | 0.0754 | |
| | *hsf-1* | 2.5907 | 0.1901 | |
| | *mtl-1* | 1.18 | 0.4223 | |
| | *hsp-70* | 1.9904 | 0.2817 | |
| *act-1* | *daf-2* | 4.6071 | 0.0871 | |
| | *daf-16* | 0.1575 | 0.9196 | |
| | *sod-3* | 0.4967 | 0.7041 | |
| | *skn-1* | 1.0828 | 0.4516 | |
| | *bec-1* | 0.1771 | 0.9067 | |
| | *aak-2* | 0.3590 | 0.7868 | |
| | *pha-4* | 6.3683 | 0.0528 | |
| | *let-363* | 22.921 | 0.0056 | Δ*atp*(-Glu) v Δ*atp*(+Glu)*: 5.6933, 0.0160<br>Δ*atp*(-Glu) v MS(-Glu)*: 7.8722, 0.0049<br>Δ*atp*(-Glu) v MS(+Glu)*: 5.8565, 0.0145<br>Δ*atp*(+Glu) v MS(-Glu): 2.1790, 0.2708<br>Δ*atp*(+Glu) v MS(+Glu): 0.1633, 0.9982<br>MS(-Glu) v MS(+Glu): 2.0158, 0.3171 |
| | *daf-9* | 2.5356 | 0.1952 | |

| **Internal control** | **Gene** | **Test statistic** | ***P* value** | **Tukey's *post-hoc* test (t statistic, *p* value) (df = 4)** |
|---|---|---|---|---|
| | *hsf-1* | 3.7575 | 0.1168 | |
| | *mtl-1* | 1.2575 | 0.4008 | |
| | *hsp-70* # | 15.594 | 0.026 | Δ*atp*(+Glu) v MS(-Glu): 4.9992, 0.0311<br>Δ*atp*(+Glu) v MS(+Glu): 0.3439, 0.9382<br>MS(-Glu) v MS(+Glu): 4.6553, 0.03765 |

# comparison of expression in animals fed on *B. s.* MS(-Glu), MS(+Glu) and Δ*atp*(+Glu) without Δ*atp*(-Glu) (for which only one biological replicate was collected).

**Appendix C**

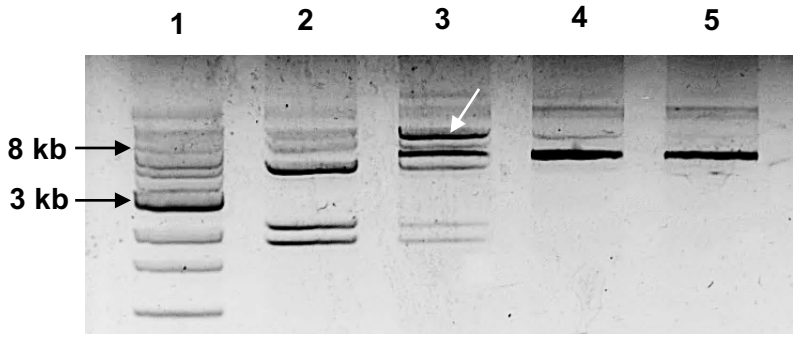



**Figure C1 *Sma*I partial digest of pDR_*unc-22* plasmid run on a 0.8% agarose gel.** Lane 1, Purple 1kb DNA ladder (NEB); Lane 2, full digestion (undiluted *Sma*I); Lane 3, 1/10 dilution of *Sma*I in Buffer J; Lane 4, 1/100 dilution of *Sma*I in Buffer J; Lane 5, undigested plasmid. The 1/10 dilution in lane 3 gave a clear band at around 8kb constituting the linearised pDR-unc vector (indicated with a white arrow).

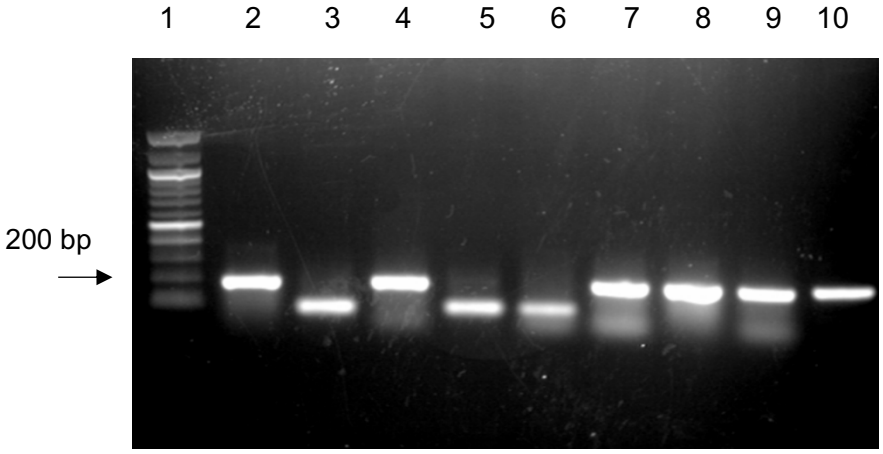



**Figure C2 Single colony PCR to confirm ligation of pDR_*unc-22* and f-Phyper-spank.** 8 colonies were selected for colony PCR using primers 1 and 3 in Table 5. 1 and were run on agarose gel (Lanes 2-9). Lane 10, control reaction without bacterial inoculation. Lane 1, purple 100 bp DNA ladder (NEB). In lanes 3, 5 and 6, the amplified fragment is not of the expected size of Phyper-spank (~200 bp) (no f-Phyper-spank ligation). Three colonies (lanes 4, 7 and 8) were selected for plasmid purification.

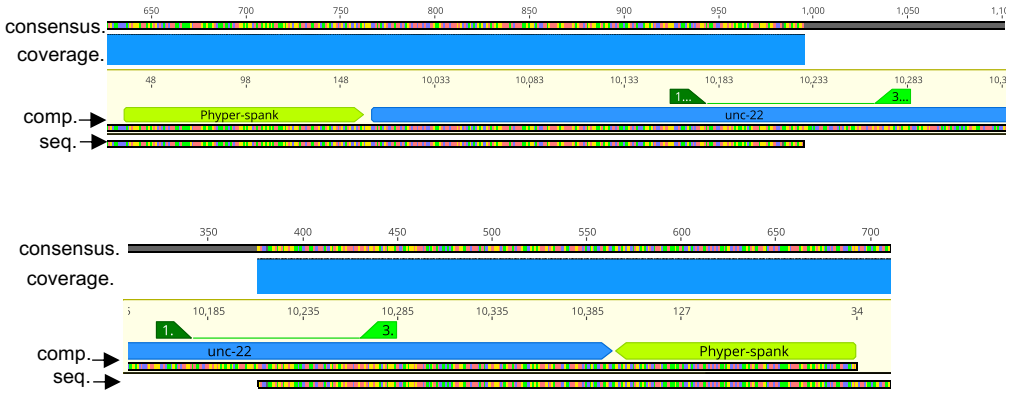



**Figure C3 Sanger sequencing to confirm the construction of pDR_*unc-22*_hs.** Shown are Geneious alignments of sequenced plasmid (seq) aligned against a composite sequence (comp.) of *unc-22* flanked by two Phyper-spank (generated in Geneious). Primers used for sequencing were primer 3 (top) and primer 1 (bottom) (Table 5. 1.).

**Table C1 qRT-PCR analysis of *unc-22* transcript levels in un-induced or IPTG-induced *B. s.* Δ*rnc*/pDR and Δ*rnc*,Δ*atp*/pDR.** Mean and standard deviation of technical triplicates for each sample were calculated. Samples were collected in biological duplicate. The difference in mean Ct (cycle threshold) of *unc-22* and the indicated internal control were calculated (ΔCt), and from this the relative abundance of *unc-22* transcripts (2^(-ΔCt)) compared to the internal control was determined.

| Strain | IPTG concentration (μM) | Replicate | Internal control | Mean Ct | St. Dev Ct | Mean Ct (*unc-22*) | St. Dev Ct (*unc-22*) | ΔCt | Relative abundance |
|---|---|---|---|---|---|---|---|---|---|
| Δ*rnc*/pDR | 0 | 1 | *sigA* | 20.3333333 | 0.26159766 | 27.28 | 0.15099669 | 6.94666667 | 0.008106716 |
| Δ*rnc*/pDR | 0 | 2 | *sigA* | 22.2833333 | 0.08736895 | 28.3533333 | 0.19295941 | 6.07 | 0.014884969 |
| Δ*rnc*/pDR | 1 | 1 | *sigA* | 21.2466667 | 0.17009801 | 17.5466667 | 0.11503623 | -3.7 | 12.99603834 |
| Δ*rnc*/pDR | 1 | 2 | *sigA* | 19.4633333 | 0.05507571 | 16.1466667 | 0.05773503 | -3.3166667 | 9.963596979 |
| Δ*rnc*/pDR | 3 | 1 | *sigA* | 16.8266667 | 0.09609024 | 13.82 | 0.11532563 | -3.0066667 | 8.037053395 |
| Δ*rnc*/pDR | 3 | 2 | *sigA* | 21.0533333 | 0.07371115 | 17.9833333 | 0.19553346 | -3.07 | 8.397733469 |
| Δ*rnc*,Δ*atp*/pDR | 0 | 1 | *sigA* | 17.1 | 0.08544004 | 22.3766667 | 0.03511885 | 5.27666667 | 0.025796754 |
| Δ*rnc*,Δ*atp*/pDR | 0 | 2 | *sigA* | 20.0533333 | 0.02516611 | 28.1766667 | 0.13012814 | 8.12333333 | 0.003586188 |
| Δ*rnc*,Δ*atp*/pDR | 1 | 1 | *sigA* | 19.68 | 0.12165525 | 18.1633333 | 0.18448125 | -1.5166667 | 2.861291865 |
| Δ*rnc*,Δ*atp*/pDR | 1 | 2 | *sigA* | 18.1633333 | 0.18448125 | 17.8133333 | 0.17785762 | -2.1566667 | 4.458834546 |
| Δ*rnc*,Δ*atp*/pDR | 3 | 1 | *sigA* | 18.8366667 | 0.08962886 | 18.0733333 | 0.12503333 | -0.7633333 | 1.697407943 |
| Δ*rnc*/pDR | 0 | 1 | *recF* | 23.1966667 | 0.10692677 | 27.28 | 0.15099669 | 4.08333333 | 0.058992145 |
| Δ*rnc*/pDR | 0 | 2 | *recF* | 24.9633333 | 0.13796135 | 28.3533333 | 0.19295941 | 3.39 | 0.095391201 |
| Δ*rnc*/pDR | 1 | 1 | *recF* | 25.0766667 | 0.09073772 | 17.5466667 | 0.11503623 | -7.53 | 184.822937 |

| Strain | IPTG concentration (μM) | Replicate | Internal control | Mean Ct | St. Dev Ct | Mean Ct (*unc-22*) | St. Dev Ct (*unc-22*) | ΔCt | Relative abundance |
|---|---|---|---|---|---|---|---|---|---|
| Δ*rnc*/pDR | 1 | 2 | *recF* | 23.9566667 | 0.28112868 | 17.8133333 | 0.17785762 | -6.1433333 | 70.68505605 |
| Δ*rnc*/pDR | 3 | 1 | *recF* | 18.9266667 | 0.06658328 | 13.82 | 0.11532563 | -5.1066667 | 34.45560218 |
| Δ*rnc*/pDR | 3 | 2 | *recF* | 24.5966667 | 0.10214369 | 17.9833333 | 0.19553346 | -6.6133333 | 97.90654039 |
| Δ*rnc*,Δ*atp*/pDR | 0 | 1 | *recF* | 17.1 | 0.08544004 | 22.3766667 | 0.03511885 | 5.27666667 | 0.025796754 |
| Δ*rnc*,Δ*atp*/pDR | 0 | 2 | *recF* | 25.8966667 | 0.0450925 | 28.1766667 | 0.13012814 | 2.28 | 0.205897754 |
| Δ*rnc*,Δ*atp*/pDR | 1 | 1 | *recF* | 24.8533333 | 0.12423097 | 18.1633333 | 0.18448125 | -6.69 | 103.2501452 |
| Δ*rnc*,Δ*atp*/pDR | 1 | 2 | *recF* | 23.9566667 | 0.28112868 | 17.8133333 | 0.17785762 | -6.1433333 | 70.68505605 |
| Δ*rnc*,Δ*atp*/pDR | 3 | 1 | *recF* | 24.0566667 | 0.20984121 | 18.0733333 | 0.12503333 | -5.9833333 | 63.2648973 |

**Table C2 Statistical comparison of *unc-22* ΔCt expression values for IPTG-induced *B. s.* Δ*rnc*/pDR and Δ*rnc*,Δ*atp*/pDR.** ΔCt values were calculated by subtracting average Ct for *unc-22* from the expression of the internal controls *sigA* and *recF* (see Table C1 above). Welch Two-sample t-test compared to control un-induced bacteria. *, $p < 0.05$

| Internal control | Strain | IPTG μM | Test statistic (df) | P value |
|---|---|---|---|---|
| *sigA* | *B. s.* Δ*rnc*/pDR(-Glu) * | 1 | 20.94 (1.37) | 0.0111 |
| *sigA* | *B. s.* Δ*rnc*/pDR(-Glu) * | 3 | 21.72 (1.01) | 0.0284 |
| *sigA* | *B. s.* Δ*rnc*,Δ*atp*/pDR(-Glu) | 1 | 5.85 (1.10) | 0.09205 |
| *sigA* | *B. s.* Δ*rnc*/pDR (-Glu) | 3 | 3.43 (1.57) | 0.1053 |
| *recF* | *B. s.* Δ*rnc*/pDR(-Glu) * | 1 | 13.64 (1.47) | 0.0159 |
| *recF* | *B. s.* Δ*rnc*/pDR(-Glu) * | 3 | 11.57 (1.41) | 0.0230 |
| *recF* | *B. s.* Δ*rnc*,Δ*atp*/pDR(-Glu) | 1 | 6.69 (1.07) | 0.0843 |
| *recF* | *B. s.* Δ*rnc*,Δ*atp*/pDR(-Glu) * | 3 | 5.56 (1.97) | 0.0321 |

**Appendix D**

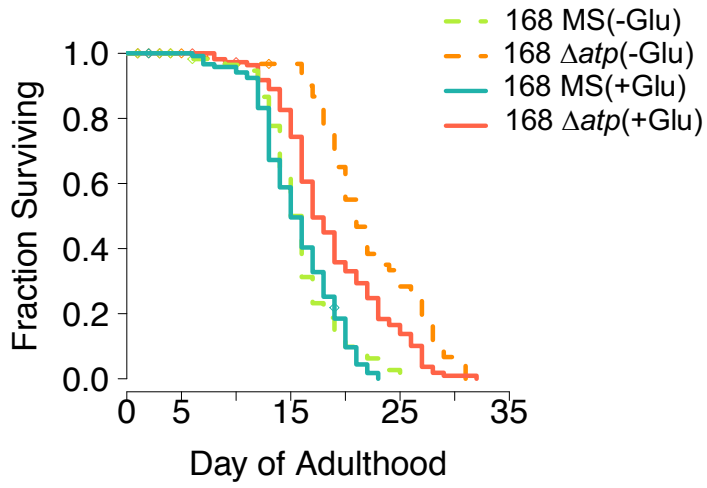



**Figure D1 *B. subtilis* 168 Δ*atp* extended the lifespan of *C. elegans* (repeat).** Lifespan of N2 individuals fed on *B. s.* 168 MS or 168 Δ*atp*(-Glu) or +Glu was monitored. Nematodes fed on *B. s.* 168 Δ*atp* lived longer than 168 MS-fed individuals both with and without glucose ($p < 0.0001$, log rank test). However, nematodes fed *B. s.* 168 Δ*atp*(-Glu) were the longest lived, showing an increase in mean lifespan of around 40% compared to those fed *B. s.* 168 MS(-Glu) ($p < 0.0001$, log rank test.

**Table D1 Summary of lifespan assays using spore-less strains *B. s.* 168 MS and 168 Δ*atp*.** The figure panels to which the experiment refers are indicated. Shading indicates experiments conducted in parallel. The mean and median lifespan and standard error values were calculated from a log rank test from samples of at least 92 individuals each. (-Glu), minus glucose; (+Glu), plus glucose 2%. C, concentrated *B. subtilis* lawn. U, unconcentrated lawn. l, lost from escape; b, bagged; v, vulval explosion. Symbols indicate comparisons between groups. *p*, log-rank test on Kaplan-Meier survival curves with Holm-Sidak multiple comparison *p* -value correction. ns, no significant difference ($p > 0.05$).

| Symbol | Figure | Strain | *B. subtilis* diet | C/U | Deaths/ total (% censored) | Censored | Mean±SEM (days) | Median (days) | Max* (days) | *p*-val / % increase ** |
|---|---|---|---|---|---|---|---|---|---|---|
| ◊ | D1 | N2 | 168 MS(-Glu) | U | 112/119 (6) | 7 (3l;3b;1v) | 15.75 ± 0.34 | 16 ± 0.28 | 22.92 | ♦ 0.7596<br>o < 0.0001<br>• < 0.0001 |
| ♦ | D1 | N2 | 168 MS(+Glu) | U | 118/122 (3) | 4 (1l;2b;1v) | 15.65 ± 0.34 | 15 ± 0.5 | 21.5 | ◊ 0.7596<br>o < 0.0001<br>• < 0.0001 |
| o | D1 | N2 | 168 Δ*atp*(-Glu) | U | 60/102 (41) | 42 (36l;4b;2v) | 21.93 ± 0.66 | 21 ± 0.77 | 29.3 | ◊ < 0.0001 / +39.2<br>♦ < 0.0001 / + 40.1<br>• 0.0005 / +17.1 |
| • | D1 | N2 | 168 Δ*atp*(+Glu) | U | 109/121 (10) | 12 (9l;2b;1v) | 18.72 ± 0.48 | 17 ± 0.61 | 27.67 | ◊ < 0.0001 / +18.9<br>♦ < 0.0001 / +19.6<br>o 0.0005 |
| ◊ | 6. 6A | N2 | 168 MS(-Glu) | U | 115/129 (11) | 14 (5l,3b,6v) | 14.39 ± 0.34 | 14 ± 0.40 | 22.08 | ♦ 0.31 / n.s.<br>o < 0.0001 |

| Symbol | Figure | Strain | *B. subtilis* diet | C/U | Deaths/ total (% censored) | Censored | Mean±SEM (days) | Median (days) | Max* (days) | *p*-val / % increase ** |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | • < 0.0001 |
| ♦ | 6. 6A | N2 | 168 MS(+Glu) | U | 118/122 (3) | 4 (2b,2v) | 14.08 ± 0.27 | 13 ± 0.36 | 19.92 | ◊ 0.31 / n.s.<br>o < 0.0001<br>• < 0.0001 |
| o | 6. 6A | N2 | 168 Δ*atp*(-Glu) | U | 101/125 (19) | 24 (14l,7b,3v) | 21.34 ± 0.47 | 21 ± 0.55 | 29.62 | ◊ < 0.0001 / +48.3<br>♦ < 0.0001 / +51.6<br>• < 0.0001 / +23 |
| • | 6. 6A | N2 | 168 Δ*atp*(+Glu) | U | 131/152 (14) | 21 (13l,5b,3v) | 17.42 ± 0.34 | 17 ± 0.44 | 25.27 | ◊ < 0.0001 / +21.1<br>♦ < 0.0001 / +24<br>o < 0.0001 |
| # | 6. 6B*** | N2 | 168 MS(-Glu) | U | 86/94 (9) | 8 (8v) | 15.77 ± 0.43 | 15 ± 0.44 | 24.44 | ¶ 0.0003 |
| ¶ | 6. 6B*** | N2 | 168 Δ*atp*(-Glu) | U | 84/100 (16) | 16 (6b; 10v) | 18.22 ± 0.4 | 18 ± 0.32 | 25.10 | # 0.0003 / +16 |

* Maximum lifespan was calculated as the mean of the top 10% longest-lived individuals in each group.

** Where the diet extended lifespan, the percentage increase in mean lifespan (%) is given.

*** > food, larger bacterial lawns in the absence of glucose.

**Table D2 Percentage of nematodes on days 2, 7 and 11 of adulthood in each zone of the NGM plate seeded with *B. subtilis* 168 MS or 168 Δ*atp*.** The relationship between lawn location (zone 1-4) and diet within each day was compared using a Pearson's Chi squared test. $\chi 2$ test statistics and *p*-values were calculated using Yates' continuity correction for 2x2 contingency tables.

| **Day 2** | **Diet (total number of individuals)** | | | | **$\chi 2$** |
|---|---|---|---|---|---|
| **Zone** | **168 MS(-Glu) (228)** | **168 Δ*atp*(-Glu) (233)** | **168 MS(+Glu) (235)** | **168 Δ*atp*(+Glu) (254)** | |
| **1** | 24.12 | 30.47 | 6.81 | 5.12 | 547.6073, $p <$ 0.0001 |
| **2** | 35.96 | 13.30 | 0.43 | 0.79 | |
| **3** | 38.16 | 12.02 | 91.06 | 67.32 | |
| **4** | 1.75 | 44.21 | 1.70 | 26.77 | |
| **Day 7** | **Diet (total number of individuals)** | | | | **$\chi 2$** |
| **Zone** | **168 MS(-Glu) (231)** | **168 Δ*atp*(-Glu) (208)** | **168 MS(+Glu) (236)** | **168 Δ*atp*(+Glu) (241)** | |
| **1** | 46.32 | 44.71 | 69.92 | 22.82 | 616.1593, $p <$ 0.0001 |
| **2** | 50.65 | 52.88 | 28.81 | 2.49 | |
| **3** | 2.16 | 0 | 0.85 | 58.09 | |
| **4** | 0.87 | 2.40 | 0.42 | 16.60 | |
| **Day 11** | **Diet (total number of individuals)** | | | | **$\chi 2$** |
| **Zone** | **168 MS(-Glu) (204)** | **168 Δ*atp*(-Glu) (174)** | **168 MS(+Glu) (211)** | **168 Δ*atp*(+Glu) (236)** | |
| **1** | 80.39 | 74.71 | 72.51 | 68.22 | 172.7643, $p <$ 0.0001 |
| **2** | 17.65 | 20.69 | 23.70 | 0 | |
| **3** | 1.47 | 2.87 | 2.370 | 23.31 | |
| **4** | 0.49 | 1.72 | 1.42 | 8.47 | |

| **Day 2 (>food)*** | **Diet (total number of individuals)** | | **$\chi$2** |
|---|---|---|---|
| **Zone** | **168 MS(-Glu) (110)** | **168 *Δatp*(-Glu) (124)** | |
| **1** | 42.72 | 80.65 | 34.2773, $p$ < 0.0001 |
| **2** | 54.54 | 14.52 | |
| **3** | 1.82 | 0 | |
| **4** | 0.91 | 4.84 | |
| **Day 7 (>food)*** | **Diet (total number of individuals)** | | $\chi$2 |
| **Zone** | **168 MS(-Glu) (106)** | **168 *Δatp*(-Glu) (119)** | |
| **1** | 84.91 | 90.76 | 1.3054, $p$ = 0.2532 |
| **2** | 14.15 | 5.88 | |
| **3** | 0.94 | 0 | |
| **4** | 0 | 3.36 | |
| * > food, more food seeded onto NGM(-Glu) plates (2.7 x the quantity) | | | |

**Table D3 The effect of the addition of more food (>food) on plate location.** Pairwise comparisons were made using Pearson's Chi square test. $\chi$2 test statistics and $p$-values were calculated using Yates' continuity correction for 2x2 contingency tables. See Table D2 for the proportion of animals in each zone.

| **Day** | **Diet comparison** | **$\chi$2** |
|---|---|---|
| 2 | 168 Δ*atp*(-Glu) v 168 Δ*atp*(-Glu) >food | 51.5, $p$ < 0.0001 |
| | 168 MS(-Glu) v 168 MS(-Glu) >food | 98.6, $p$ < 0.0001 |
| 7 | 168 Δ*atp*(-Glu) v 168 Δ*atp*(-Glu) >food | 42.97, $p$ < 0.0001 |
| | 168 MS(-Glu) v 168 MS(-Glu) >food | 65.827, $p$ < 0.0001 |

**Table D4 Raw pharyngeal pumping counts min$^{-1}$ of *C. elegans* fed on *B. subtilis* 168 MS or 168 Δ*atp*.** Data were collected on days 1, 4 and 15 of adulthood across two independent trials. Shaded cells indicate animals that were outside of bacteria and were therefore excluded from the analysis.

| Diet | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) | 168 Δ*atp* (+Glu) | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) | 168 Δ*atp* (+Glu) | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) | 168 Δ*atp* (+Glu) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day of adulthood | | | | | | | | | | | |
| Animal | 1 | | | | 4 | | | | 15 | | | |
| 1 | 257 | 288 | 237 | 254 | 211 | 240 | 219 | 185 | 0 | 9 | 0 | 97 |
| 2 | 246 | 304 | 267 | 274 | 261 | 257 | 219 | 249 | 32 | 77 | 0 | 101 |
| 3 | 258 | 279 | 280 | 277 | 275 | 242 | 263 | 189 | 15 | 95 | 0 | 47 |
| 4 | 265 | 293 | 272 | 266 | 227 | 261 | 201 | 208 | 0 | 94 | 62 | 0 |
| 5 | 273 | 280 | 248 | 223 | 234 | 275 | 254 | 232 | 32 | 95 | 35 | 0 |
| 6 | 280 | 296 | 268 | 260 | 198 | 268 | 253 | 200 | 0 | 0 | 0 | 7 |
| 7 | 267 | 280 | 210 | 277 | 271 | 258 | 253 | 206 | 42 | 89 | 11 | 90 |
| 8 | 267 | 286 | 263 | 282 | 249 | 293 | 276 | 257 | 10 | 0 | 0 | 91 |
| 9 | 261 | 279 | 275 | 249 | 273 | 257 | 228 | 227 | 26 | 90 | 0 | 20 |
| 10 | 254 | 278 | 262 | 259 | 229 | 265 | 256 | 238 | 0 | 113 | 0 | 0 |
| 11 | 283 | 114 | 273 | 290 | 213 | 257 | 261 | 268 | 0 | 71 | 51 | 0 |
| 12 | 281 | 295 | 285 | 92 | 206 | 259 | 244 | 245 | 42 | 90 | 45 | 12 |
| 13 | 260 | 209 | 286 | 262 | 258 | 283 | 219 | 229 | 50 | 100 | 0 | 95 |
| 14 | 290 | 299 | 297 | 323 | 246 | 284 | 264 | 298 | 0 | 65 | 61 | 0 |
| 15 | 289 | 302 | 297 | 305 | 268 | 299 | 230 | 272 | 21 | 12 | 38 | 51 |
| 16 | 285 | 320 | 299 | 298 | 247 | 280 | 239 | 190 | 0 | 89 | 0 | 96 |

| Diet | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) | 168 Δ*atp* (+Glu) | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) | 168 Δ*atp* (+Glu) | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) | 168 Δ*atp* (+Glu) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day of adulthood | | | | | | | | | | | |
| Animal | 1 | | | | 4 | | | | 15 | | | |
| 17 | 289 | 301 | 301 | 308 | 213 | 245 | 268 | 277 | 45 | 0 | 0 | 0 |
| 18 | 259 | 331 | 280 | 301 | 304 | 261 | 235 | 253 | 20 | 70 | 40 | 0 |
| 19 | 293 | 332 | 288 | 297 | 244 | 291 | 212 | 278 | 34 | 56 | 74 | 84 |
| 20 | 300 | 324 | 287 | 291 | 270 | 259 | 207 | 268 | 0 | 91 | 0 | 78 |
| 21 | 295 | 300 | 283 | 270 | 259 | 260 | 258 | 180 | | | | |
| 22 | 270 | 215 | 293 | 300 | 218 | 274 | 260 | 280 | | | | |
| 23 | 283 | 301 | 257 | 302 | 270 | 259 | 274 | 276 | | | | |
| 24 | 268 | 306 | 270 | 289 | | 280 | 258 | 253 | | | | |
| Mean | 273.88 | 298.76 | 274.08 | 280.74 | 235.92 | 266.96 | 243.79 | 239.92 | 18.45 | 65.30 | 20.85 | 43.45 |
| St. Dev | 14.89 | 16.71 | 21.22 | 23.25 | 53.64 | 15.89 | 22.14 | 35.05 | 18.20 | 38.49 | 26.52 | 42.93 |
| Median | 260.75 | 286.00 | 266.00 | 264.00 | 216.75 | 257.75 | 225.75 | 207.50 | 17.5 | 83 | 0 | 33.5 |
| IQR | 286.00 | 304.00 | 287.25 | 299.00 | 268.50 | 280.00 | 260.25 | 269.00 | 32.5 | 46.75 | 41.25 | 90.25 |

**Table D5 Significance of differences in mean pharyngeal pumping rates in *C. elegans* fed on *B. s.* 168 MS or 168 Δ*atp*.** See Table D4 for raw data.

| Day | One-way ANOVA | Tukey's HSD *post-hoc* test | | | |
|---|---|---|---|---|---|
| 1 | $F = 8$, $p < 0.0001$ | | 168 Δ*atp* (-Glu) | 168 Δ*atp* (+Glu) | 168 MS (-Glu) |
| | | 168 Δ*atp*(+Glu) | 0.0141 | - | - |
| | | 168 MS(-Glu) | 0.0002 | 0.6184 | - |
| | | 168 MS(+Glu) | 0.0002 | 0.6416 | 1 |
| 4 | $F = 8.7565$, $p < 0.0001$ | **Games-Howell *post-hoc* test** | | | |
| | | 168 *Δatp*(+Glu) | 0.0084 | - | - |
| | | 168 MS(-Glu) | 0.0122 | 0.9324 | - |
| | | 168 MS(+Glu) | 0.0009 | 0.9677 | 0.9962 |
| 15 | **Kruskal-Wallis** | **Dunn's *post-hoc* test** | | | |
| | $\chi 2 = 17$, $p = 0.0007$ | 168 *Δatp*(+Glu) | 0.1181 | - | - |
| | | 168 MS(-Glu) | 0.0018 | 0.1114 | - |
| | | 168 MS(+Glu) | 0.0018 | 0.1114 | 0.9056 |

**Table D6 Descriptive statistics and within-day statistical comparisons of body area data for *C. elegans* fed on *B. s.* 168 MS or 168 Δ*atp* with and without glucose.** Body area was measured on day 3 and 6 of adulthood. One-way ANOVA with Tukey's *post-hoc* test.

| **Day** | **Condition** | **N** | **Mean area ± St. Dev.** | **Test statistic, *p*-value** | ***p*-value (Tukey's *post-hoc* test)** | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) | 168 Δ*atp* (+Glu) |
| 3 | 168 MS (-Glu) | 30 | 101415 ± 7152 | $F(3,124) = 26.6612$ $p < 0.0001$ | - | - | - | - |
| | 168 Δ*atp* (-Glu) | 33 | 92146 ± 8471 | | < 0.0001 | - | - | - |
| | 168 MS (+Glu) | 33 | 104517 ± 6580 | | 0.3101 | < 0.0001 | - | - |
| | 168 Δ*atp* (+Glu) | 32 | 91946 ± 5854 | | < 0.0001 | 0.9994 | < 0.0001 | - |
| 6 | 168 MS (-Glu) | 29 | 124136 ± 11694 | $F(3,114) = 22.5709$ $p < 0.0001$ | - | - | - | - |
| | 168 Δ*atp* (-Glu) | 30 | 124027 ± 9581 | | 1 | - | - | - |
| | 168 MS (+Glu) | 29 | 115416 ± 10010 | | 0.0058 | 0.0066 | - | - |
| | 168 Δ*atp* (+Glu) | 30 | 105817 ± 8354 | | < 0.0001 | < 0.0001 | 0.0017 | - |

**Table D7 Thrashing rate in liquid of *C. elegans* fed on *B. s.* 168 MS or 168 Δ*atp* in the absence (-Glu) or presence (+Glu) of glucose.** One-way ANOVA (with Welch's correction for day 2 and 8) followed by Tukey's HSD (day 5) or Games-Howell (for day 2 and 8) *post-hoc* test. N = 40. Data were collected by Said (2018).

| **Day** | ***B. s.* diet** | **Mean thrashes ± St. Dev.** | **Test statistic, *p*-value** | ***Post-hoc* test *p*-value** | | |
|---|---|---|---|---|---|---|
| | | | | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) |
| 2 | 168 MS(-Glu) | 58.58 ± 9.68 | $F(3,85.16) = 53.67$, $p < 0.0001$ | - | - | - |
| | 168 Δ*atp*(-Glu) | 87.43 ±13.89 | | < 0.0001 | - | - |
| | 168 MS(+Glu) | 57.85 ± 7.41 | | 0.98 | < 0.0001 | - |
| | 168 Δ*atp*(+Glu) | 67.98 ± 8.09 | | < 0.0001 | < 0.0001 | < 0.0001 |
| 5 | 168 MS(-Glu) | 48.78 ± 8.90 | $F(3, 156) = 70.81$, $p < 0.0001$ | - | - | - |
| | 168 Δ*atp*(-Glu) | 73.13 ± 11.6 | | < 0.0001 | - | - |
| | 168 MS(+Glu) | 45.85 ±10.03 | | 0.5500 | < 0.0001 | - |
| | 168 Δ*atp*(+Glu) | 65.65 ± 8.78 | | < 0.0001 | 0.0050 | < 0.0001 |
| 8 | 168 MS(-Glu) | 42.8 ± 6.79 | $F(3, 85.85) = 57.84$, $p < 0.0001$ | - | - | - |
| | 168 Δ*atp*(-Glu) | 65 ± 9.91 | | < 0.0001 | - | - |
| | 168 MS(+Glu) | 41.8 ± 6.61 | | 0.91 | < 0.0001 | - |
| | 168 Δ*atp*(+Glu) | 49.68 ± 8.1 | | 0.0006 | < 0.0001 | < 0.0001 |

**Table D8 Paraquat survival of *C. elegans* fed on *B. s.* 168 MS or 168 Δ*atp*.** Summary statistics and pairwise comparisons of survival in the presence of 500 mM paraquat on the indicated day of adulthood. The number of deaths after 7 hrs of exposure is indicated. Only events or right-censored data were present. Data were analysed with the log-rank test on Kaplan-Meier survival curves (Holm *post-hoc* multiple correction). N = 90, 3 independent replicates. Data were collected by Said (2018).

| **Day** | ***B. s.* diet** | **Deaths/total (% censored)** | **Median survival time** | **Log-rank test *p*-value** | | |
|---|---|---|---|---|---|---|
| | | | | 168 MS (-Glu) | 168 Δ*atp* (-Glu) | 168 MS (+Glu) |
| 2 | 168 MS(-Glu) | 84/90 (7) | 5 | - | - | - |
| | 168 Δ*atp*(-Glu) | 34/90 (62) | - | < 0.0001 | - | - |
| | 168 MS(+Glu) | 88/90 (2) | 4.5 | 0.317 | < 0.0001 | - |
| | 168 Δ*atp*(+Glu) | 79/90 (12) | 5 | 0.098 | < 0.0001 | 0.007 |
| 5 | 168 MS(-Glu) | 88/90 (2) | 3 | - | - | - |
| | 168 Δ*atp*(-Glu) | 37/90 (59) | - | < 0.0001 | - | - |
| | 168 MS(+Glu) | 90/90 (0) | 2.5 | 0.3293 | < 0.0001 | - |
| | 168 Δ*atp*(+Glu) | 82/90 (9) | 4 | 0.0138 | < 0.0001 | 0.0009 |
| 8 | 168 MS(-Glu) | 90/90 (0) | 3 | - | - | - |
| | 168 Δ*atp*(-Glu) | 59/90 (34) | 5 | < 0.0001 | - | - |
| | 168 MS(+Glu) | 90/90 (0) | 2 | 0.0031 | < 0.0001 | - |
| | 168 Δ*atp*(+Glu) | 90/90 (0) | 3 | 0.5601 | < 0.0001 | 0.0005 |

**Table D9 Proportion of *C. elegans* fed on *B. s.* 168 MS and 168 Δ*atp* alive after heat shock at 37 °C, on day 2, 5 and 8 of adulthood.** Exposure time was 6 hrs (day 2), 4 hrs (day 5) or 2 hrs (day 8). Data were collected from 4 independent replicates (30 individuals per replicate). Data were collected by Said (2018).

| | | 168 MS(-Glu) | | | 168 Δ*atp*(-Glu) | | | 168 MS(+Glu) | | | 168 Δ*atp*(+Glu) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Day** | | **2** | **5** | **8** | **2** | **5** | **8** | **2** | **5** | **8** | **2** | **5** | **8** |
| **Replicate** | **1** | 20.00 | 10.00 | 26.67 | 46.67 | 30.00 | 43.33 | 13.33 | 0.00 | 16.67 | 33.33 | 26.67 | 30.00 |
| | **2** | 30.00 | 26.67 | 20.00 | 63.33 | 36.67 | 36.67 | 26.67 | 6.67 | 23.33 | 30.00 | 33.33 | 26.67 |
| | **3** | 30.00 | 20.00 | 40.00 | 43.33 | 40.00 | 46.67 | 13.33 | 16.67 | 20.00 | 36.67 | 20.00 | 26.67 |
| | **4** | 26.67 | 16.67 | 43.33 | 50.00 | 26.67 | 53.33 | 20.00 | 13.33 | 30.00 | 26.67 | 13.33 | 46.67 |
| **Average** | | 26.67 | 18.33 | 32.50 | 50.83 | 33.33 | 45.00 | 18.33 | 9.17 | 22.50 | 31.67 | 23.33 | 32.50 |
| **St. Dev.** | | 4.71 | 6.94 | 11.01 | 8.77 | 6.09 | 6.94 | 6.38 | 7.39 | 5.69 | 4.30 | 8.61 | 9.57 |

**Table D10 Statistical analysis of differences in the proportion of *C. elegans* surviving heat shock.** See Table D9 for raw data.

| Day | *B. s.* diet | Welch ANOVA Test statistic, *p*-value | *Post-hoc* test *p*-value | | |
|---|---|---|---|---|---|
| | | | 168 MS(-Glu) | 168 Δ*atp*(-Glu) | 168 MS(+Glu) |
| 2 | 168 MS(-Glu) | $F(3,6.5) = 10.619$, $p = 0.0066$ | - | - | - |
| | 168 Δ*atp*(-Glu) | | 0.0206 | - | - |
| | 168 MS(+Glu) | | 0.2606 | 0.0052 | - |
| | 168 Δ*atp*(+Glu) | | 0.4601 | 0.0491 | 0.0588 |
| 5 | 168 MS(-Glu) | $F(3,6.6) = 7.5293$, $p = 0.0152$ | - | - | - |
| | 168 Δ*atp*(-Glu) | | 0.065 | - | - |
| | 168 MS(+Glu) | | 0.355 | 0.010 | - |
| | 168 Δ*atp*(+Glu) | | 0.804 | 0.329 | 0.160 |
| 8 | 168 MS(-Glu) | $F(3,6.5) = 6.964$, $p = 0.0191$ | - | - | - |
| | 168 Δ*atp*(-Glu) | | 0.326 | - | - |
| | 168 MS(+Glu) | | 0.455 | 0.011 | - |
| | 168 Δ*atp*(+Glu) | | 1 | 0.257 | 0.375 |

**Table D11 DAF-16::GFP localisation in *C. elegans* strain TJ356 fed on *B. s.* 168 MS or 168 Δ*atp*.** Counts of number of individuals with each of the three types of localisation are given, as well as the percentage. *E. coli* refers to strain OP50-1. The *E. coli*(-Glu) control was exposed to 37°C heat shock for 15 minutes. Data was collected across two independent trials. Pairwise Fischer's Exact test with FDR *p* value correction was used to determine pairwise significance (excluding the positive control group). The Monte Carlo method was used for estimating significance. *p* values below 0.05 were considered significant.

| Diet | DAF-16::GFP localisation; count (percentage) | | | N | Pairwise Fischer's Exact test *p* value | | |
|---|---|---|---|---|---|---|---|
| | Cytoplasmic | Intermediate | Nuclear | | 168 MS(-Glu) | 168 Δ*atp*(-Glu) | 168 MS(+Glu) |
| *E. coli*(-Glu) control | 0 | 0 | 23 (100) | 23 | - | - | - |
| *B. s.* 168 MS(-Glu) | 48 (100) | 0 | 0 | 48 | - | - | - |
| *B. s.* 168 Δ*atp*(-Glu) | 38 (77.55) | 4 (8.16) | 7 (14.29) | 49 | 0.0031 | - | - |
| *B. s.* 168 MS(+Glu) | 46 (97.87) | 0 | 1 (2.13) | 47 | 0.4947 | 0.0244 | - |
| *B. s.* 168 Δ*atp*(+Glu) | 48 (90.57) | 2 (3.77) | 3 (5.66) | 53 | 0.2426 | 0.2870 | 0.4422 |